Understanding the effects of temperature on raspberry physiology and gene expression profiles

PhD Thesis
Tek Prasad Gotame
January 2014
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Tek Prasad Gotame

Thesis

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy at Aarhus University, Faculty of Science and Technology, Department of Food Science, Aarhus University, Denmark
Tek Prasad Gotame

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For the memory of my mother
This Ph.D. thesis has been submitted to Aarhus University as a partial fulfilment of the requirements of the degree of Doctor for Philosophy. My main supervisor has been Dr Lillie Andersen, Department of Food Sciences, Aarhus University. Dr Karen K Petersen, Department of Food Sciences, Aarhus University and Dr Julie Graham have been co-supervisors. Dr Hanne Lindhard Pedersen acted as main supervisor during the first one third of the PhD period before she left the Department. The study was conducted from August 2010 to December 2013, and was primarily based at the Department of Food Sciences in Aarslev, Denmark. From April 2012, I stayed for four months at the James Hutton Institute, Scotland, UK to analyse gene expression profiles using microarray and real-time qRT-PCR under the supervision of Dr Julie Graham and Dr Danny W Cullin to whom I am grateful for invaluable advice and collaboration.

The thesis deals with the effect of high temperature stress on physiology and flowering behaviour in association with the possibility of tunnel production of organic and conventional raspberries in Danish conditions. Originally it was planned to include transnational trials of the ClimaFruit project (Interreg IVB North Sea Region, EU Programme Project ID: 35-2-05-09, which has financed the PhD-study) to study the effect of climate on fruit yield and quality in raspberries in six transnational sits, but this trial could not be included owing to lack of uniformity in the trial set up in such factors as cultivars, planting geometry and fertilizer applications. Therefore the study on organic versus conventional fertilization has been included with a focus on fruit yield and quality under Danish conditions. Its aims were to understand the heat stress mechanism in commercial raspberries and to demonstrate the possibility and opportunity for sustainable production of organic raspberries of high quality. The thesis is a culmination of three years of work including planning, laboratory work, field experiments, data measurement, analysis and preparation for publications.

In this thesis, I present 13 chapters. Chapter 1 deals with justification of the problem being explored. Chapter 2 presents raspberry botany and differences in cultivars. In Chapter 3, a review is carried out on dormancy regulation in woody perennials. Reviews of the effect of temperature on flowering behaviours are discussed in Chapter 4 while the effects of high temperature and the principle of photosynthetic efficiency is presented in Chapter 5. How
plants sense heat is described with a diagram adapted from the literature in Chapter 6. Factors affecting raspberry fruit quality are reviewed and discussed in Chapter 7. Chapter 8 briefly presents materials and methods used, and Chapter 9 include one published article. Chapters 10 and 11 include manuscripts to be submitted for publication. In Chapter 12, key general results from the research are discussed. Conclusions are drawn and future perspectives are presented in Chapter 13.

I owe a debt of gratitude to many people who have helped and encouraged me during my Ph.D. study and the project financing the PhD-study Interreg IVB North Sea Region, EU Programme Project ID: 35-2-05-09. I would like to thank my supervisors Lillie Andersen, Karen K Petersen and Julie Graham for their guidance, help and constructive criticisms. Thank you Carl-Otto Ottosen for your invaluable advices and corrections during my greenhouse and climate chamber experiment. I am also grateful to Dr Danny W Cullin, the James Hutton Institute, UK for patiently guiding me through the genetic background and molecular work completed as part of the study and sharing with me his extensive knowledge.

I would particularly like to thank Connie, Elin, Annette, Karin, Helle and Elizabeth for helping me in field and lab work. I am thankful to the Horticulture Research Division, Nepal Agriculture Research Council (NARC) for facilitating my study leave. And last but not least, I thank my dear family, my wife Bandana Sharma and lovely son Lakchya Gotame for their patience and love. Thank to my daughter, Rojina, you have joined in our family in later part of my PhD thesis, you motivated me to accomplish the Thesis work.
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Abbreviations

ABA  Abscisic acid
BLASTn  Basic Logical Alignment Search Tool for Nucleotide
BLASTx  Basic Logical Alignment Search
C: N  Carbon nitrogen ratio
CAM  Crasulian Acid Metabolism
cDNA  Complementary DNA
$C_t$  Threshold cycle
D/N  Day/night
DNA  Deoxyribonucleic acid
DW  Dry weight
EC  Electrical conductivity (mS cm$^{-1}$)
ESTs  Expressed sequence tags
ETF  Electron transferring flavoprotein
FADH2  Flavin adenine dinucleotide dehydrogenase
FAO  Food and Agriculture Organization
$F_m$  Maximal fluorescence of dark adapted leaf
$F_o$  Minimal fluorescence of dark adapted leaf
FST  Institute of Food Science and Technology
$F_v$  Variable fluorescence of dark adapted leaf
Fv/Fm  Maximum photochemical efficiency of PSII
FW  Fresh Weight
GA  Gibberellic acid
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
gDNA  Genomic DNA
$g_s$  Stomatal conductance (mmol m$^{-2}$ s$^{-1}$)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>JHI</td>
<td>James Hutton Institute</td>
</tr>
<tr>
<td>LHCII</td>
<td>Light harvesting complex II</td>
</tr>
<tr>
<td>Mbp</td>
<td>Mega base pair</td>
</tr>
<tr>
<td>MIP</td>
<td>membrane intrinsic proteins (MIPs)</td>
</tr>
<tr>
<td>MIQC</td>
<td>Minimum Information for Publication of Quantitative Real-Time PCR Experiments</td>
</tr>
<tr>
<td>mRNA</td>
<td>Microsomal ribonucleic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate dehydrogenase</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIPs</td>
<td>plasma membrane intrinsic proteins</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>QA</td>
<td>Primary electron acceptor quinine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity (%)</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reaction Oxygen species</td>
</tr>
<tr>
<td>Rubisco</td>
<td>Ribulose-1,5-bisphosphatase carboxylase/oxygenase</td>
</tr>
<tr>
<td>RuBP</td>
<td>Ribulose bisphosphate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TA</td>
<td>Titratable acid</td>
</tr>
<tr>
<td>TIP</td>
<td>Tonoplast intrinsic proteins</td>
</tr>
<tr>
<td>TSS</td>
<td>Total soluble solid</td>
</tr>
<tr>
<td>UPL</td>
<td>Universal Probe Library</td>
</tr>
<tr>
<td>VPD</td>
<td>Vapour pressure deficit (kPa)</td>
</tr>
</tbody>
</table>
Summary

Raspberry (Rubus idaeus L) is a high-value horticultural crop due to its taste, nutritional quality and health benefits. Protected cultivation of raspberries in greenhouses or plastic tunnels is increasing for both season extension and quality production reasons across European countries. Manipulation of flowering and fruiting is essential for extension and year-round production of raspberries. Increased temperature in summer months may change their flowering behaviour and results in reduced fruit yields and quality. Exposure to elevated temperatures above the physiological optimum induces up- and down-regulation of genes resulting in morphological, anatomical, physiological and biochemical changes in tissues. Photosynthesis is one of the key physiological processes affected by high temperature stress while down- or up-regulation of gene profiles is an effect at the molecular level. The project was aimed at finding out the effects of high temperature stress on photosynthetic efficiency (Fv/Fm), flowering and fruiting behaviour and gene expression profiles of commercial raspberry cultivars. It was also aimed at investigating the effect of organic and conventional production methods on fruit yield and quality of three annual- and four biannual-fruited cultivars for two consecutive seasons in Danish conditions.

In the first part of study, experiments were carried out in greenhouse and climate chambers with elevated temperature regimes from a standard level of 20 °C to 27, 32 or 37 °C for a seven day period during flower initiation on photosynthetic efficiency (Fv/Fm), chlorophyll concentration, flowering and fruiting behaviours and gene expression profiles. Negative responses to heat stress were reflected in a decreased midday Fv/Fm in all five cultivars, while there was a remarkable difference in chlorophyll pigments and flowering behaviour among cultivars. There was a decline in the efficacy of photosystem II under elevated temperature regimes at midday and partial recovery in the evening in ‘Polka’. An extended cold-storage period suppresses lateral shoot formation and promotes the number of flower buds per lateral in ‘Autumn Bliss’ and ‘Fall Gold’. Moreover, heat stress enhances early flowering in ‘Autumn Bliss’ and delays in ‘Autumn Treasure’ to some extent, indicating distinct cultivar differences. In commercial production, this information may be useful for manipulating and optimizing fruit production in glasshouses and outside in warmer regions. In the second part of study, a custom Rubus microarray in combination with real-time qRT-PCR were used to understand and identify the candidate genes involved in the heat stress response of four
annual-fruited raspberry cultivars. A 10 °C elevation in temperature altered the expression of 40 genes (38 were down-regulated and two were up-regulated) among the four annual-fruited raspberry cultivars. Two aquaporin genes (PIP1 and TIP2) were down-regulated in ‘Autumn Bliss’ but up-regulated in ‘Autumn Treasure’, ‘Polka’ and ‘Erika’. Validation by real-time qRT-PCR indicated subtle gene expression differences suggesting a mild response to heat stress. In the third part of study, three annual- and four biannual-fruited raspberry cultivars were evaluated in conventional and organic tunnels in Danish conditions for berry yield, size and quality attributes over two consecutive years.

There were field x cultivar, field x year and cultivar x year interaction for yield in annual-fruiting cultivars whereas biannual-fruited cultivars showed cultivar x field x year interaction. ‘Autumn Bliss’ produced the highest yield (4.6 kg m⁻¹ row) in both conventional and organic fields and was consistent over the years and field conditions among three annual-fruiting cultivars. Similarly ‘Glen Fyne’ produced the highest yield (11.1 kg m⁻¹ row) in organic field in 2013 among biannual-fruiting cultivars. ‘Octavia’ produced the largest fruit size (5.8 g) in 2012. There were also field x cultivar, field x year and cultivar x year interactions for total soluble solid (TSS) in annual-fruiting cultivars and field x year and cultivar x year interaction in biannual-fruiting cultivars. The TSS was highest in ‘Fall Gold’ (9.7 °Brix) in 2011 and in ‘Tulameen’ (11.5 °Brix) in 2013 in an organic field. Titratable acid (TA) was lower in 2013 compared to 2012 in biannual-raspberries. The highest citric acid was observed in ‘Autumn Bliss’ (138.0 µg mg⁻¹ DW) in a conventional field. It was found that citric and malic acids were decreased in 2012 compared to 2011 in annual-fruited raspberries. Similarly, glucose and fructose were higher in 2012 compared to 2011. Sugars were not affected by fields but year variations were observed in annual-fruiting cultivars. The highest glucose (127.1 µg mg⁻¹ DW) and fructose (146.3 µg mg⁻¹ DW) were measured in 2012 but were not different between cultivars. Glucose and fructose were different between fields and a higher concentration was found in 2013 than 2012 in biannual-fruited cultivars. ‘Tulameen’ has higher sucrose (130. 4 µg mg⁻¹ DW) but statistically similar with ‘Glen Ample’. From yield and quality analysis of all cultivars, ‘Autumn Bliss’ was the best cultivar for autumn production in organic field since it had high yield with large fruit and moderate TSS: TA ratio, and was consistent across the years and fields. ‘Octavia’ and ‘Glen Fyne’ are the promising cultivars for summer production in Danish conditions.
Sammendrag

Hindbær (*Rubus idaeus* L) er en afgrøde med høj værdi på grund af bærrenes smag, ernæringsmæssige kvalitet og sundhedsfremmende egenskaber. Interessen for en længere dyrkningsperiode og bedre kvalitet ved produktion af hindbær i væksthus eller plastik tunnel er stigende i hele Europa. For at opnå en længere sæson er styring af blomstring og bærdannelse helt nødvendig. Øget temperatur i sommerperioden kan have indflydelse på blomsterdannelsen og resultere i mindre udbyte og ringere kvalitet. Udsættes hindbærplanter for temperaturer over deres fysiologisk optimum, kan det bevirke op- og nedregulering af gener og dermed resultere i ændringer i planten af morfologisk, anatomisk, fysiologisk og biokemisk art. Fotosyntesen er en af de nøgleprocesser, som påvirkes af høj temperatur stress, hvor op- og nedregulering af gener påvirker på det molekylære plan. Nærværende projekt har til formål at undersøge effekten af høj temperatur stress på fotosynteseeffektivitet (*F*<sub>v</sub>/F<sub>m</sub>), blomstring og bærdannelse, samt gene ekspression i kommersielt dyrkede hindbær sorter. Formålet var ligeledes at undersøge effekten af dyrkning økologisk i forhold til konventionelt på udbyte og bærkvalitet i 3 efterårshindbær og 4 sommerhindbær sorter over 2 sammenhængende år under danske forhold.

Den første del af studierne blev gennemført i væksthus og klimakammer med forhøjede temperaturer fra 20 °C til 27, 32 eller 37 °C gennem en 7-dages periode under blomsterdannelsen. Effekten af forhøjet temperatur på fotosynteseeffektivitet (*F*<sub>v</sub>/F<sub>m</sub>), klorofyl koncentration, blomstring, udbyte og gene ekspression blev undersøgt. I alle fire sorter af sommerhindbær blev der fundet negativ effekt af forhøjet temperatur på F<sub>v</sub>/F<sub>m</sub> midt på dagen, medens der var en markant forskel mellem sorterne mht. klorofyl koncentration og blomstring. Der blev observeret et fald i effektiviteten af fotosystem II midt på dagen under forhøjet temperatur og en delvis forbedring senere på dagen i sorten Polka. Hindbær opbevares på køl før plantning. En forlængelse af køleopbevaringen af hindbærrene før plantning reducerede antallet af sideskud, men fremmede mængden af blomsterknopper per sideskud i sorterne Autumn Bliss og Fall Gold. Derudover gav en forhøjet temperatur en tidlig blomstring i Autumn Bliss og forsinkede blomstringen i Autumn Treasure i nogen grad, hvilket viser forskelle mellem sorterne. I kommerciel produktion vil denne nye viden kunne bruges til at manipulere og optimere hindbærproduktionen i væksthus og på friland i varmere regioner.
I anden del af studierne blev et microarray udviklet specifikt til *Rubus* anvendt i kombination med real-time qRT-PCR med henblik på at forstå og identificere, hvilke gener, der er involveret i planternes respons på forhøjet temperatur i 4 sorter af efterårshindbær. En forhøjelse af temperaturen med 10 °C ændrede ekspressionen af 40 gener (38 blev nedreguleret og 2 opreguleret) hos de 4 sorter. To aquaporin gener (*PIP1* og *TIP2*) blev nedreguleret i Autumn Bliss, men opreguleret i Autumn Treasure, Polka og Erika. Valideringen vha. qRT-PCR viste diskrete forskelle i gen ekspression, hvilket indikerede en mild respons på varme stress.

I den tredje del af studierne blev 4 sorter af sommer- og 3 af efterårshindbær evalueret mht. udbytte, bærstørrelse og –kvalitet ved konventionel og økologisk dyrkningsmetode i tunnel under danske betingelser over 2 år. Der blev fundet vekselvirkning mellem dyrkningsmetode og sort, dyrkningsmetode og år, sort og år i udbytterne i efterårshindbær, medens i sommerhindbær var der vekselvirkning mellem sort, dyrkningsmetoder og år. Af efterårshindbærrene gav sorten Autumn Bliss det højeste udbytte (4,6 kg/m række) i både konventionel og økologisk dyrkning konsekvent over begge år. Tilsvarende gav sommersorten Glen Fyne det højeste udbytte (11,1 kg/m række) i både konventionel og økologisk dyrkning i 2013. Sorten Octavia gav de største bær (5,8 gram/bær) i 2012. Der var også vekselvirkninger mellem dyrkningsmetode og sorter, dyrkningsmetoder og år, samt sort og år mht. bærkvalitet som totalt opløseligt tørstof (TSS) i efterårshindbær. Tilsvarende var der vekselvirkning mellem dyrkningsmetode og år, samt sort og år i sommerhindbærerne.

TSS var størst i Fall Gold (9,7 °Brix) i 2011 og i Tulameen (11,5 °Brix) i 2013 ved økologisk dyrkningsmetode. Titrérerbarsyre (TA) var lavere i 2013 end i 2012 i sommerhindbærsorterne. Det største indhold af citronsyre blev fundet i sorten Autumn Bliss (145,7 µg/mg DW) under konventionel dyrkning i 2011. Indholdet af citronsyre og æblesyre faldt i 2012 i forhold til 2011 i efterårshindbærerne. Derimod var indholdet af glukose og fruktose større i 2012 i sammenligning med 2011. Sukkerindholdet blev ikke påvirket af dyrkningsmetode, men der var effekt af dyrkningsåret i efterårshindbær. Størst glukose (127,1 µg/mg DW) og fruktose indhold (146,3 µg/mg DW) blev fundet i 2012 uden forskel mellem sorterne. Glukose og fruktose indholdet var forskelligt mellem dyrkningsmetoder og en højere koncentration blev set i 2013 end i 2012 i sommerhindbær. Sorten Tulameen har et højt sukrose indhold (130,4 µg/mg DW) tilsvarende som i sorten Glen Ample. Sorten Autumn Bliss vil være en velegnet sort til efterårsdyrkning, idet udbyttet er højt og med god smag, og sorterne Octavia og Glen Fyne er de mest lovende sorter til sommerproduktion af hindbær under danske betingelser.
Chapter 1: Introduction

Most raspberry (*Rubus idaeus* L) production is concentrated in the temperate areas of the world with a total area and production estimated at 100,556 ha and 543,421 tonnes respectively (FAO, 2011). The largest proportion comes from Europe with 445,918 tonnes of production including 73 tonnes from Denmark and 16,761 tonnes from the United Kingdom. Although most of the production is concentrated in northern and central Europe, raspberry production is expanding to warm areas of southern Europe (Oliveira et al., 1996; Graham and Jennings, 2009). Extending the season of production and double cropping in high tunnels and greenhouses are also of growing interest to the raspberry industry. Manipulation of flowering and fruiting is essential for out-of-season production. Use of early and late fruiting cultivars, summer pruning of spring shoots, use of polyethylene tunnels and greenhouses are commercial practices used to extend the season and for year-round production of raspberries (Oliveira et al., 1996; Carew et al., 2000a; Oliveira et al., 2002; Pitsioudis et al., 2002; Dale et al., 2005).

The Intergovernmental Panel on Climatic Change (IPCC) has reported that global temperature is rising by 0.2 °C per decade (IPCC, 2007). Whether related to this or not, a heat wave in Europe was experienced from June to mid-August 2003 with summer temperatures raised by 3 to 5 °C (Fink et al., 2004) and maximum temperatures of 35 to 40 °C being recorded in southern and central Europe (Beniston and Diaz, 2004; Schar and Jendritzky, 2004). Such extreme weather events may re-occur in the future and become a limiting factor for perennial woody species. Increased temperature in summer months may change their flowering behaviour and result in reduced fruit yields. High temperatures above the normal optimum (e.g. >10 - 15 °C to optimum) are sensed as stress in many plant species (Kotak et al., 2007; Wahid et al., 2007). Stress occurs when the temperature rises beyond a threshold level for a period sufficient to damage plant growth and development.

Exposure to elevated temperatures induces up- and down-regulation of genes resulting in morphological, anatomical, physiological and biochemical changes in plant tissues (Zhang et al., 2005). One of the key physiological processes affected by high temperature stress is photosynthesis by altering the function and ultrastructure of the organelles, stomata regulation, pigment concentration and biosynthesis, protein and metabolites and enzyme
activities (Berry and Bjorkman, 1980; Wahid et al., 2007). For example, Calvin cycle reaction and photosynthetic electron transport are highly susceptible to high temperature stress and therefore decrease photosystem II (PSII) yield (Salvucci and Crafts-Brandner, 2004a). Rubisco activase becomes less effective in maintaining ribulose-1,5-bisphosphate carboxylase/oxygenase in the catalytically active state at temperatures above 40 °C (Salvucci and Crafts-Brandner, 2004a, b). It has also been reported that inhibition of photosynthesis by heat stress (i.e. at 30 - 42 °C) is due to decreasing rate of ribulose-1,5-bisphosphate regeneration caused by disruption of electron transport activity, and specifically inactivation or damage of the oxygen evolving complex of photosystem II. Moreover, high temperature decreases the concentration of chlorophyll pigments, alters the ratio of chlorophyll a to chlorophyll b and chlorophyll to carotene depending on the thermotolerance capacity of the plant species (Camejo et al., 2005; Guo et al., 2006; Efeoglu and Terzioglu, 2009; Almeselmani et al., 2012).

Earlier studies have used chlorophyll fluorescence as a non-invasive tool to determine the level of heat tolerance in raspberry cultivars and a protocol has been developed (Molina-Bravo et al., 2011). Changes in variable to maximum chlorophyll fluorescence (Fv/Fm) could be a good indicator for screening and understanding heat resistant cultivars. Heat sensible raspberry cultivars showed the lowest Fv/Fm at midday (Molina-Bravo et al., 2011). Other climatic factors such as solar radiation, daylight, soil and air temperature also impact on growth, flowering, yield and chemical composition of raspberry cultivars as reported by Williams, (1959); Hudson, (1959); Prive et al., (1993a); Stafne et al., (2000; 2001); Carew et al., (2003); Sonsteby and Heide, (2009; 2010; 2012) and Remberg et al., (2010).

However, the effect of high temperature stress for a short period (about a week) at the flower initiation stage on chlorophyll fluorescence, chlorophyll pigments, and subsequent flowering and fruiting behaviours of commercial raspberry cultivars is not documented. It is imperative to better understand the response of commercial raspberry cultivars to the elevated temperature regimes on photosynthetic efficiency and flowering phenomenon. Knowledge of the mechanism of high temperature stress in commercial raspberry cultivars for wider recommendations has importance for advancing our understanding. Therefore, an experiment was carried out to pin point the effects of short period heat stress during flower initiation on photosynthetic efficiency, flowering behaviour and gene expression profiles of selected annual-fruiting raspberry cultivars under greenhouse and climate chamber conditions. A field
experiment was also conducted to study the effect of organic and conventional field management on berry yield and quality in three annual- and four biannual-fructing raspberry cultivars.

1.1 Hypotheses

The following hypotheses were formulated.

1. High temperature stress decreases $F_v/F_m$ faster in heat sensitive than heat tolerant annual-fruiting raspberries (Article 1, Figure 1 and Figure 2).

2. High temperature stress decreases chlorophyll pigment concentration and alters their ratios (Article 1, Figure 3).

3. Flower induction is inhibited whereas flower development is advanced by high temperature stress in annual-fructing raspberry cultivars (Article 1, Table 2, Figure 4).

4. When raspberries are exposed to high temperature for a short period (24 h), heat responsive genes are up-regulated or down-regulated (Manuscript 2, Figure 1, 2, 3 and 4).

5. Raspberry fruit yield and berry size depend on cultivar, year and production system (Manuscript 3, Table 1 and 2, Figure 2, 3 and 4).

6. It is possible to produce quality organic raspberries in Danish conditions similar to a conventional field (Manuscript 3, Table 3, 4, 5, 6 and 7).

1.2 Objectives

The objectives of this study were to determine the effects of high temperature stress during flower initiation on the photosynthetic efficiency and chlorophyll pigments of five annual-fruiting raspberries ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, ‘Fall Gold’ and ‘Polka’. The effects of high temperature stress on flowering and fruiting behaviour of these cultivars were also investigated. It was also aimed to determine if chlorophyll fluorescence can be used as a screening criterion for high-temperature sensitivity in annual-fructing raspberry cultivars. We used Rubus Agilent 60k microarrays to identify the effect of high temperature stress for a short period (24 h) on gene expression profiles of ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’ and ‘Polka’ and also to determine the linkage map of heat responsive candidate genes.

It was also aimed to demonstrate the possibility of high quality organic raspberry production in Danish conditions. Therefore evaluation was carried out to find out the effect of organic and conventional production methods on berry yield and size, sugar and acid compositions in three annual- and four biannual-fructing raspberry cultivars for two consecutive years.
Chapter 2: Raspberry botany

2.1 Origin and taxonomy

The Ide Mountains of Turkey are considered to be the origin of raspberries (Jenning, 1988) and China is the centre of diversity (as reviewed by Graham and Jennings, 2009). Raspberries (*Rubus idaeus* L.) belong to the family *Rosacea* and genus *Rubus* and have diploid (2n = 2x = 14) chromosomes with a small genome (275 Mbp). About 600 - 800 species are reported from blackberry, red raspberry and black raspberry. 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.) are important for commercial production (Figure 2.1) (Bushway et al., 2008; Graham and Jennings, 2009).

![Botanical classification of raspberries and blackberries](source: Bushway et al., 2008).

Raspberries are perennial in habit but above ground canes die after two summers i.e. they have short lived woody shoots on a perennial crown and root system (Hudson and Williams,
New canes of the first year are called primocanes and are produced every year from underground roots or basal cane buds (Figure 2.2). The cane shows a sigmoidal growth pattern (Carew et al., 2000b). When they are in the second year of growth, the canes are called floricanes (Smith et al., 2007).

Figure 2.2. Vegetative parts and growth habit of raspberry plant (source: Smith et al., 2007).

Flowers are 0.5 - 1.5 cm in size containing five sepals and petals with one short hypanthium, 60 - 80 stamens, and a gynoecium with 60 - 80 ovaries (Graham et al., 2007). Fruit development follows three stages: in the first stage, the growth is accelerated and it continues until the second stage where the growth slows down. Embryo and seeds develop and mature in this stage. Ripening and senescence occur in the last stage (plateau phase). Pre-harvest fruit qualities such as texture, flavour, pigments, nutritional values and secondary metabolites change in the last stage. Fruit develops within 30 to 36 days after pollination (Graham et al., 2007; Graham and Jennings, 2009). Bumble bees and honey bees are the major pollinators in the raspberry field, therefore these bees are required for maximizing fruit production in the early and late season under tunnel conditions. In open field conditions, bees searching for food are stop on flowers on their way and perform adequate pollination.
2.2 Raspberry breeding program

There are 30 *Rubus* breeding programmes in 19 countries, almost all of which are in Europe and North America (Graham and Jennings, 2009). The James Hutton Institute (JHI, Scotland, UK) is one of the leading raspberry breeding institutions along with East Mailing Research Centre, UK. It has been reported that the Scottish bred cultivar ‘Glen Ample’, released in the mid-1990’s along with ‘Tulameen’ and recently the new cultivar ‘Octavia’ dominate the UK market and acreage due to their desirable fresh market characteristics. In Denmark, ‘Glen Ample’ and ‘Autumn Bliss’ are the dominate cultivars (Håndbog for Frugt- og bæravlere 2013. ISBN 978-87-89051-01-07)

2.3 Classification of cultivars

Raspberry cultivars are divided into two groups; annual-fruiting (synonymous: primocane; ever-bearing; autumn, fall bearing) cultivars and biennial-fruiting (synonymous: floricane; summer) bearing cultivars according to the flowering and fruiting habits (Keep, 1988; Carew et al., 2000b; 2003).

2.3.1 Annual-fruiting cultivars

Annual-fruiting cultivars have a one year cycle during which vegetative growth, dormancy breaking, flower initiation and flowering are completed (Keep, 1988; Sonsteby and Heide, 2009). Annual-fruiting cultivars develop many canes but are shorter in height (1 to 1.8 m) compared to biannual-fruiting cultivars (2.5 to 3 m) because apical flowers suppress further growth in height in annual cultivars (Keep, 1988).

In annual-fruiting raspberries, new canes are produced in early spring (March-April) from the previous year’s root sucker or dormant crown and begin continued vegetative growth (Figure 2.3A). In the first year during spring and summer, the vegetative growth of shoots follows a sigmoid pattern and stops in summer with few terminal flowers. Flower initiation occurs early in the season during June-July when canes are actively growing and having 20 - 22 leaves as in ‘Autumn Bliss’ (Carew et al., 2001). Dormancy of the canes and crown is induced in early autumn when temperature is decreasing (10 - 15 °C) and the photoperiod is shortening (< 10 h) and enters into endodormancy in the early winter which lasts for 8 - 10 weeks (William, 1960). Dormancy releases in winter (around February) and new canes emerge in spring for flowering. Flowering occurs in these new canes during late summer.
(August) to early autumn (September). After flowering and fruiting, the shoots die at frost and the life cycle is completed.

### 2.3.2 Biannual-fruiting cultivars

Biannual-fruiting cultivars have a two years growth cycle during which they complete induction and breaking of bud dormancy, vegetative growth, flowering and fruiting (Figure 2.3B) (Williams, 1960; Sonsteby and Heide, 2008; Sonsteby et. al., 2009). Canes develop from root suckers or adventitious root buds (dormant crown) and resume vegetative growth and continue until late summer in the first year. Shoot growth decreases toward the end of summer (Hudson and Williams, 1961). Shoots die at frost in the winter in the second year (Williams, 1960; Sonsteby and Heide, 2008).

![Figure 2.3. Growth cycle of A) annual and B) biannual-fruiting raspberries (Adopted from Heide and Sonsteby, 2011).](image)

Flower initiation occurs in the terminal bud after elongation of the shoot has ceased, for example, in ‘Mailing Primose’. In contrast, flower initiation occurs in a terminal bud in some cultivars such as ‘Lloyd George’ even though the shoots are still elongating (Williams, 1959). In general, flower initiation and dormancy occur during early autumn followed by flower differentiation in mid-autumn (September-October) at short day conditions. Floral primordium appears from top 10 to 15 lateral buds as in ‘Mailing Primose’ (Williams, 1959).
According to Williams (1959), both short days and low temperatures promote dormancy induction in biannual-fruiting raspberries. Axillary buds are formed in the first year flower buds in basipetal succession at the 15 - 20 leaf stage as in ‘Malling Promise’ (Williams, 1960) and ‘Glen Ample’ (Sonsteby and Heide, 2008). It is reported that the first axillary buds with flower primordia can be observed in the region of five to fifteen buds below the apex and initiate progressively down the shoot. The earliest indication of flower initiation is a broadening of the apical meristem and elongation of the growing point (Williams, 1960). These axillary buds grow out in spring to produce laterals bearing fruits in the second year. Along with the rise in temperature in spring, vegetative growth resumes and produces flowers in early June on the previous year’s cane. The floricanes produce heavy fruits in late summer and die in winter and therefore complete the growth cycle. At the same time, new shoots are formed from root suckers and axillary root buds that maintain the perennial habit of the raspberry plant.

Some cultivars (e.g. ‘Lloyd George’) produce a few fruits at the tip of the cane at the end of the first year (autumn) while the rest of the canes are still in the vegetative stage. The same cane continues to produce fruits in summer. Similarly ‘Glen Moy’ initiates flowers earlier than the normal season and produces few fruits at the tip of the cane during autumn in the first year (reviewed by Carew et al, 2000b). This type of behaviour is sometimes defined as tip bearing habits.

In summary, the time of flower initiation determines whether a cultivar is in the annual- or biannual- fruiting group. The difference between these major two types of raspberry growth cycle is due to the fruiting cycle of the plant. In addition, these cultivars are different in their responses to temperature requirements and the day length for flowering.
Chapter 3: Dormancy regulation in raspberries

3.1 Dormancy

Dormancy is defined as an inability of a plant to initiate growth or a temporary suspension of growth under favourable conditions (Lang, 1987). There are three kinds of physiological processes in dormancy: ecodormancy, paradormancy and endodormancy. Ecodormancy is also called imposed dormancy or quiescence and is a condition when buds are dormant as a result of external factors unfavourable to growth (e.g. temperature, water supply, photoperiod etc.) (Mazzitelli et al., 2007). During ecodormancy, growth is stopped due to unfavourable conditions which can occur before or after endodormancy. Paradormancy is also called correlative inhibition and is the condition when buds cannot grow due to an inhibitory effect of other parts within the plant itself, for example, dormancy of lateral buds due to the dominance of the terminal shoots. In this case, bud growth is suppressed by an apical dominant organ such as adjacent leaves. Apical dominance (paradormancy) is strong in raspberries and results in the typical unbranched growth habit of the cane until growth is terminated by flower induction (Mazzitelli et al., 2007). It has also been reported that after formation of the bud during the first summer of growth, the buds enter into a period of paradormancy due to the inhibitory effect of the adjacent leaves and apical dominance. Therefore when a plant is in paradormancy, growth resumes if it is exposed to favourable conditions (Lang et al., 1987).

Endodormancy is the stage when buds are dormant because of internal physiological factors which stop the growth even under favourable external conditions and is also called rest or true dormancy (Lang et al., 1987). Endodormancy is controlled by the bud itself and requires a specific amount of chilling (e.g. <7 °C) in order to remove the condition. During late autumn, buds progressively enter into endodormancy. In autumn, the dormancy begins and becomes stronger (deeper) until November-December depending on the plant species and cultivars. For example, the raspberry cultivar ‘Latham’ reached deepest dormancy during mid October in the USA while ‘Glen Moy’ and ‘Glen Clova’ reached maximum dormancy at the end of October in the UK (as reviewed by Carew et al., 2001). However, this difference could have been either due to environmental or genetic factors. Abscisic acid (ABA) is believed to play a regulatory role for the onset of dormancy leading to cold acclimation (as reviewed by Pagter et al., 2008a). It has also been reported that ABA is accumulated in dormant buds and
decreases after being exposed to effective chilling temperatures. Moreover, bud dormancy is regulated by the balance between growth inhibitors like ABA, and growth promoting substances like cytokinin and gibberellin.

3.2 Dormancy regulation, cold treatment and vernalization in annual-fruiting raspberries

Raspberry plants enter into dormancy in the late autumn when temperature is decreasing and the photoperiod is shortening. According to Williams (1959), both short days (<10 h) and low temperature (<16 °C) are responsible for dormancy induction in raspberries. Palonen, (2006) reported that dormancy was deeper after incubation at 20 °C than at 4 °C, and short days induced deeper dormancy than long day conditions. Generally, a short photoperiod gives a signal for initiation of hardening and dormancy in deciduous plants. Leaves are the sensors for the short day response. The transition from ecodormancy (quiescence) to endodormancy is usually completed by October-November. At the termination of dormancy, there is an increase in growth promoters (gibberellic acid, cytokinin) relative to inhibitors (ABA).

A specific amount of chilling is required to terminate or break the rest period (endodormancy) and restore the bud’s ability to expand and resume growth again. Cold treatment breaks the dormancy and starts the development of fruiting laterals in the subsequent spring in raspberries as in many other woody deciduous species (Mazzitelli et al., 2007). By subjecting the raspberry plants to a low temperature of about 3 °C, bud dormancy can be broken in about four weeks after which they respond rapidly to warm conditions (10 - 15 °C) and grow out into fruiting laterals (Hudson and Williams, 1961).

Chilling and cold treatment affect raspberry vegetative and reproductive behaviours. Carew et al., (2001) reported that chilling decreased the number of nodes and leaves in annual-fruiting raspberries. For example, when ‘Autumn Bliss’ was exposed to chilling at 7 °C for 10 weeks after lifting in October, the number of nodes were 22 compared to 36 in plants receiving no chilling treatment in the UK (Carew et al., 2001). Similarly, when ‘Polka’ was grown at 6 °C for 7 weeks before being transferred to 24 °C, plant height and the number of leaves decreased but dormant buds increased compared to plants grown at 24 °C from the beginning (Sonsteby and Heide, 2009). Cane height at terminal flowering also decreased as chilling duration was increased in ‘Autumn Bliss’ (Carew et al., 2001).
Temperate woody plant species require a certain period of cold treatment (<7 °C for 800 to 1500 h) to promote flowering. This specific promotional effect of flowering by prolonged exposure to cold temperature is called vernalization (Michaels and Amasino, 2000). In annual-fruiting raspberries the effect of cold temperatures for a certain period is required (Takeda, 1993; Carew et al., 2001; Dale et al., 2003; 2005). During vernalization, cold temperatures do not cause plants to initiate floral primordia but create the capacity for subsequent flowering (Heide and Sonsteby, 2011). Vernalization advances the transition period from vegetative to reproductive stage and reduces node formation before flowering. For example, non-chilled ‘Heritage’ plants remained vegetative for over 7 months while plants chilled for 750 units flowered within four months (Takeda, 1993). The role of growth promoters like gibberellin (GA) and cytokines during transition from vegetative to reproductive stage has been reported. GAs are acting synergistically with cytokinins during flower induction in raspberries (Vasilakakis et al., 1979). It has been reported that cytokinin levels were 23% greater in cold treated than the non-cold treated plants. Similarly, GA activity was 60% greater at 20 than at 10 nodes in cold treated annual-fruiting plants (Vasilakakis et al., 1979). Moreover, cold treated flower-induced plants had 100% greater GA than non-cold treated, non-flower-induced plants at the 20 node stage.

Low temperature treatment has been reported to have an inconsistent effect on the production of flowering laterals and fruit yield in raspberry cultivars. For example, ‘Erika’ produced a higher yield while ‘Autumn Treasure’ produced a lower yield in vernalized plants compared to plants from 18 °C (Sonsteby and Heide, 2012). Dale et al., (2005) reported that cold stored plants reached 5% harvest 80 days earlier in ‘Autumn Britten’ and produced a yield two times greater than the non-cold stored plants. It has also been reported that plants cold stored (<7 °C) for 6 weeks produced a higher yield compared to those that had not been cold stored. Takeda, (1993) and Carew et al., (2001) explained that adventitious and basal buds elongated faster, produced fewer nodes and flowered earlier when annual-fruiting cultivars received chilling treatment.

In summary, cold treatment increases the vegetative growth rate and enhances flowering but decreases the number of leaves and nodes in the shoot. Therefore, low temperature exposure prior to shoot growth is beneficial and promotes flower bud initiation in annual-fruiting raspberries.
Chapter 4: Effect of temperature on flowering in raspberries

Climate factors such as temperature, photoperiod, light intensity and soil moisture all affect vegetative and reproductive performance of raspberry cultivars (Prive et al., 1993a,b). Temperature and photoperiod are the major regulating environmental factors for flower initiation, flowering and dormancy in raspberries (Williams, 1960). On the other hand, annual- and biannual-fruiting cultivars show distinct responses to temperature and photoperiod for flowering as detailed in studies by Hudson, (1959), Williams, (1959; 1960) and Sonsteby and Heide, (2009; 2010; 2012). Nonetheless, temperature effects are inconsistent within and between annual-and biannual cultivars. For example, biannual-fruiting cultivars require low temperatures (<15 °C) for growth cessation and floral initiation while low temperature is not necessarily required to initiate floral primodia in annual-fruiting cultivars, but vernalization promotes the flower induction process (as reviewed by Heide and Sonsteby, 2011). The main and interaction effects of temperature and photoperiod on flowering habits of annual-and biannual-fruiting cultivars are discussed below.

4.1 Annual-fruiting cultivars

The time of flower initiation is dependent on the temperature as shown in detailed studies of ‘Autumn Bliss’ by Carew et al., (2000a; 2003). They reported that time to flowering was shortest (110 days) at 22 °C in vernalized ‘Autumn Bliss’ while it was 160 days at 13 °C. It has also been found that when temperature increases above or decreases below 22 - 24 °C, time-to-anthesis decreased and flowering was delayed (Carew et al., 2001; 2003). Therefore, the optimum temperature for most of the annual-fruiting raspberries is an average diurnal temperature of 16 - 24 °C but some cultivars like ‘Polka’ can flower and fruit even at 30 °C (Sonsteby and Heide, 2009; Heide and Sonsteby, 2011). Reports suggest that in order to increase flowering laterals and early fruits, annual-fruiting cultivars need to be grown at 20 - 25 °C during the season. In contrast, ‘Autumn Treasure’ delayed flowering above 20 °C (Sonsteby and Heide, 2009). Advanced flowering, fruiting and ripening with a higher yield was also reported in ‘Erika’ and ‘Polka’ growing at 25 °C compared to plants growing at 15 °C (Sonsteby and Heide, 2012). Moreover, ‘Polka’ flowered only at the tip of the main shoot
when temperature was below 12 °C because most of the lateral buds remained dormant (Sonsteby and Heide, 2009).

Photoperiod also has an effect on flowering of annual-fruiting raspberries. For example, Carew et al., (2003) found that intermediate photoperiod promoted early flowering and long photoperiod delayed flowering. However, ‘Mailing Promise’ ceased growth and induced dormancy at an intermediate temperature (15.5 °C) and short photoperiod (at 9 h) while long photoperiod (at 14 h) promoted continuous growth (Williams, 1959).

At temperatures of 22 - 25 °C with intermediate photoperiod of 12 h, flower initiation was enhanced in annual-fruiting cultivars (Carew et al., 2003). These cultivars initiate flowers even at temperatures of 16 - 30 °C in long day conditions, but at low temperature and short photoperiod initiated buds remain dormant (Heide and Sonsteby, 2011).

In summary, earliness in annual-fruiting cultivars is dependent on the temperature exposure. Moreover, temperature has two effects on flowering; earliness and extension of the flowering period. In general, an increase in temperature up to 24 °C results in an increased rate of vegetative growth and advances in flowering in most of the annual-fruiting raspberries. However, there is still a debate on the role of temperature on flower formation in annual-fruiting raspberry.

4.2 Biannual-fruiting cultivars

In biannual-fruiting raspberries, flower initiation and flowering is promoted by a combination of low temperature and short day conditions (Williams, 1959; 1960; Sonsteby and Heide, 2008). In both natural habitats and with cultivated raspberries, growth continues towards the end of the season (late summer) at a decreasing rate and completely stops and enters dormancy in mid-September. The initiation of flower buds in biennial raspberries has an upper temperature limit at approximately 15 ºC (Williams, 1960; Sonsteby and Heide, 2008). After the breaking of dormancy and vernalization, the canes produce flowers at intermediate temperatures (12 - 15 ºC). Flower initiation occurs only in short days with a critical photoperiod of 15 h, while, at temperatures ≤ 12 ºC, floral initiation also takes place in long days. In contrast to flowering, vegetative growth is promoted by high temperatures and long days and increases with increasing temperature. Similarly, a warm temperature (21 °C)
induces vigorous vegetative growth and a low temperature (10 °C) stops cane elongation regardless of photoperiod (i.e. 9 h or 14 h) in ‘Malling Promise’ (Williams, 1959) while growth ceases at 15.5 °C in short day conditions (<12 h). It has also been reported that ‘Malling Promise’ continues to grow and remains vegetative at 21 °C in both long and short day conditions for 18 months giving a height up to 7 m. ‘Glen Ample’ shows growth cessation and floral initiation two weeks earlier at 15 °C compared to those grown at 18 °C, while growth was nearly doubled at 21 °C compared to 15 °C (Sonsteby and Heide, 2008).

Shoot elongation and leaf number were also enhanced by increasing temperature from 9 to 21°C giving a plant height of 85 to 350 cm in ‘Glen Ample’ as observed by Sonsteby and Heide, (2008). They observed that the floral initiation was delayed at 15 °C compared to 9 and 12 °C and no flowers were initiated at 18 °C even under short day conditions.

In summary, the period of flower induction in biannual raspberries is temperature dependent but the response is modified by photoperiod. Low temperatures (<15 °C) and long photoperiod (about 15 h) are required for growth cessation and floral initiation. Lower temperatures and longer photoperiod are necessary for flower initiation in biannual-fruiting cultivars compared to annual-fruiting cultivars.
Chapter 5: Photosynthetic efficiency and high temperature stress

5.1 Principle of chlorophyll fluorescence

When light energy is absorbed by chlorophyll molecules (excited state), there are four possible mechanisms for energy dissipation. They are 1) photosynthesis (photochemistry), 2) heat dissipation (photochemical quenching), 3) energy transfer to another molecule and 4) re-emission of photons (chlorophyll fluorescence). These processes occur in competition; if there is an increase in the efficiency of one process, there is a decrease in the yield of the others (Figure 5.1) (Maxwell and Johnson, 2000; Leipner, 2007; Baker, 2008).

![Diagram of light phenomenon in a chlorophyll molecule](Source: Leipner, 2007)

It has been reported that a small fraction of the excitation energy (1 - 2% of total light) is dissipated as fluorescence. When light is exposed on a leaf, fluorescence rises from the ground state value ($F_o$) (Photosystem II; PSII reaction centres are in ‘open’ state and quinone acceptor ($Q_A$) is maximally oxidized) to its maximum value ($F_m$) (PSII reaction centres are in ‘closed’ state and $Q_A$ is maximally reduced). The $Q_A$ is the first electron acceptor of PSII. Once $Q_A$ accepts an electron, it cannot accept another until it passes the first to a subsequent electron carrier (closed state). If a leaf is dark adapted for about 15 - 30 min and applied with a saturating flash (about 8000 μmol m$^{-2}$ s$^{-1}$ for 0.6 - 1 s), fluorescence may rise from the minimum value ($F_o$) to its maximum value ($F_m$) (Maxwell and Johnson, 2000; Leipner, 2007). The difference between $F_m$ and $F_o$ is termed as variable fluorescence ($F_v$). The photosynthetic efficiency or the maximum quantum yield of $Q_A$ reduction, i.e. PSII photochemistry, is calculated by $F_v/F_m = (F_m-F_o)/F_m$ (Baker and Rosenqvist, 2004; Baker, 2008). A decrease in $F_v/F_m$ is due to a decrease in $F_m$ and/or an increase in $F_o$. In healthy non-
stressed leaves, the $F_v/F_m$ value is close to 0.83 (Baker, 2008), while Sharma et al., (2012) reported up to 0.840 (greenhouse grown) to 0.834 (climate chamber) in wheat, and 0.82 - 0.83 in raspberry (Paper I, Chapter 9). A decrease in $F_v/F_m$ indicates the light harvesting complex is more or less damaged or inactivated (Maxwell and Johnson, 2000). If the fluorescence yield is high, less energy is emitted as heat or used in photochemistry. Therefore by measuring the amount of chlorophyll fluorescence, the efficiency of photosynthesis can be predicted.

Chlorophyll fluorescence provides insights into the ability of a plant to tolerate environmental stress and the level of damage to the photosynthetic apparatus (Maxwell and Johnson, 2000). Reduction and suppression in photosynthetic rate occur due to biotic and abiotic stresses that decrease fluorescence emission of the leaf (Baker and Rosenqvist, 2004). Many studies have been carried out to measure the effects of environmental stresses on plants such as drought, temperature, nutrient deficiency, salt stress etc. (Table 5.1). Changes in the $F_v/F_m$ ratio could also be a good indicator for screening high temperature resistant cultivars in perennial woody fruits (Kadir et al., 2007; Molina-Bravo et al., 2011). Many reports have used the maximum and minimum chlorophyll fluorescence as a monitoring tool for heat induced changes to help to predict the critical temperature for PSII inactivation and dysfunction.

**Table 5.1.** Some examples of the application of $F_v/F_m$ as a monitoring tool to detect abiotic stress.

<table>
<thead>
<tr>
<th>Abiotic stress</th>
<th>Crops</th>
<th>References</th>
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<tbody>
<tr>
<td>Low temperature stress</td>
<td>White spruce</td>
<td>Binder and Fielder, 1996</td>
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<td></td>
<td>Maize</td>
<td>Andrews et al., 1995; Fracheboud et al., 1999</td>
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<td></td>
<td>Douglas fir seedlings</td>
<td>Perks et al., 2001</td>
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<td>Rice</td>
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<td></td>
<td><em>Hydrangea</em></td>
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<td>High temperature stress</td>
<td>Potato</td>
<td>Havaux, 1993</td>
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<td></td>
<td>Grapes</td>
<td>Kadir et al., 2007</td>
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<td></td>
<td>Tomato</td>
<td>Willits and Peet, 2001</td>
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<td></td>
<td>Raspberry</td>
<td>Molina-Bravo et al., 2011</td>
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<td></td>
<td>Wheat</td>
<td>Mathur et al., 2010, 2011; Sharma et al., 2012</td>
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<td>Janka et al., 2013</td>
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<td>Salt stress</td>
<td>Maize</td>
<td>Shabala et al., 1998</td>
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<td>Abiotic stress</td>
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<td>Aluminium stress</td>
<td>Sorghum</td>
<td>Peixoto et al., 2002</td>
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<td></td>
<td>Citrus</td>
<td>Pereira et al., 2000</td>
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Most of these references were cited as reviewed by Baker and Rosenqvist, 2004.

5.2 Modulated fluorescence measurement and limitations/pitfalls

Most of the chlorophyll fluorescence measurements are carried out using a pulse modulated fluorometer with the leaf positioned in a known state (Baker and Rosenqvist, 2004). But the reasons for abiotic stress-induced decreases in $F_v/F_m$ are often complex (Baker, 2008) and there are errors associated with measurement which mislead the information of PSII efficiency. As it is easy to generate data with chlorophyll fluorescence, there is also an equal chance of generating meaningless data (Maxwell and Johnson, 2000). Therefore precautions should be taken in the design and execution of experiments to ensure accurate, precise and informative data.

Furthermore, the detection of chlorophyll fluorescence emissions is sensitive to the distance and angle between the leaf and the end of the fiberoptic cable of the fluorometer, the intensity of the measuring beam, and the leaf chlorophyll content (Logan et al, 2007) but chlorophyll fluorescence values are relative and the leaf chlorophyll content might not be an important factor. It has also been reported that the presence of pubescence, leaf surface features, the thickness of the leaf, waxes or anthocyanin content affects the strength of the chlorophyll fluorescence signal. Except in crassulacean acid metabolism (CAM) plants, fluorescence measured from an upper exposed side of a leaf provides an accurate indicator of the abiotic stress conditions (as reviewed by Longan et al., 2007). Dark adaptation is performed using special dark-adaptation clips. These clips are attached to the leaf and allow measurements at a uniform distance non-invasively. The time required for dark adaptation should be selected to a certain minimum to generate reliable and replicable data. In these conditions, fluorescence can be a powerful tool to study photosynthetic performance especially when coupled with other non-invasive tools such as absorption spectroscopy, gas exchange analyses, and infrared thermography (Baker, 2008). Maxwell and Johnson, (2000) have also mentioned that application of chlorophyll fluorescence should be combined with other techniques such as gas exchange measurement to give reliable data of plant response under high temperature stress conditions.
5. 3 Mechanism of heat damage of photosystem II

One of the key physiological processes affected by high temperature stress is photosynthesis. High temperature stress effects the process of photosynthesis by altering the function and ultrastructure of the organelles, stomata regulation, pigment concentration and biosynthesis, and protein and enzyme activities (Berry and Bjorkman, 1980; Wahid et al., 2007). There are three major high temperature stress sensitive sites in the photosynthetic apparatus; PSII, ATPase and the carbon assimilation process (Allakhverdiev et al., 2008). The decrease in photosynthetic efficiency at elevated temperature is a result of a disruption of the functional integrity of PSII (Berry and Bjorkman, 1980; Camejo et al., 2005). The major features are inactivation of PSII and thylakoid disorganization with an increase in $F_o$ as a function of temperature (as reviewed by Baker and Rosenqvist, 2004). High temperature stress affects photosynthetic performance because Rubisco activase is thermally unstable (Feller et al., 1998) and the electron transport chain is inhibited in high temperature stress conditions (Mathur et al., 2011). The repair mechanism of PSII (D1-protein) is also damaged due to the generation of reactive oxygen species (ROS), which reduces carbon fixation (Allakhverdiev et al., 2008). At high temperature (35 - 45 °C), thylakoid membranes become leaky (Sharkey, 2005). It has also been reported that an inhibition of the enzymes of the carbon cycle, activity of the water splitting complex, leaf gas exchange properties and electron transport are possible reasons for a decrease of the thylakoid membrane fluidity. Yamane et al., (1998) and Enami et al., (1994) reported that high temperature stress dissociates the Mg$^{2+}$ stabilizing protein from the PSII reaction centre complex and it releases as an electron (Mg$^+$$)$. Rubisco activase stops to release sugar phosphates and therefore inactivate Rubisco activities (Salvucci and Crafts-Bradner, 2004b). Such stress also affects membrane stacking, membrane integrity, ion conductivity and phosphorylation activity (Zhang and Sharkey, 2009). Changes in the ultrastructure of thylakoid membranes above 40 °C cause dissociation of the LHCII chlorophyll a/chlorophyll b proteins from the PSII core complex (Tang et al., 2007). This results in a decrease in the assimilation rate due to high dark respiration and, as the carbon yield is decreasing, there may be a depletion of carbohydrate reserves in the plant after certain stress periods.

An increased temperature above 38 °C for about a week decreased the maximum photochemical efficiency of PSII ($F_v/F_m$), primary photochemistry ($F_v/F_o$), and the performance index (PI), and increased minimum fluorescence ($F_o$) in chrysanthemum as
reported by Janka et al., (2013). They have also reported that at 39°C, F_v/F_m decreased by 14% and F_o increased by 25% compared to a control.

5.4 Effects of high temperature on chlorophyll pigments

High temperature stress also affects the concentration and ratios of chlorophyll pigments and their composition depending on the thermotolerance capacity of the plant species (Camejo et al., 2005; Guo et al., 2006; Efeoglu and Terzioglu, 2009) because chlorophyll biosynthesis is reduced or impaired and degraded (Reda and Mandoura, 2011). In heat tolerant plant species, the chlorophyll a to chlorophyll b ratio increased and the chlorophyll to carotenoids ratio decreased (Camejo et al., 2005). Fully developed leaves showed more degradation of chlorophyll a and b in high temperature stress when compared to developing leaves (Karim et al., 1999).

One of the most important carotenoid pigments in leaf is xanthophyll which is found in light harvesting antenna complexes. It plays an important role in light harvesting and photoprotection (Misra et al., 2006). The xanthophyll cycle (the reversible inter-conversion of two particular carotenoids, violaxanthin and zeaxanthin) is involved in photoprotection and carotenoids of the xanthophyll family have been shown to stabilize and photo-protect the lipid phase of the thylakoid membranes (Sharkey, 2005). In high temperature stress conditions, the xanthophylls including violaxanthin, antheraxanthin and zeaxanthin are partitioned between the light harvesting complexes and lipid phase of the thylakoid membranes (Adams and Demmig-Adams, 1992).

5.5 Photosynthesis study in raspberry: an example

As in other plant species, very low or very high temperatures above the physiological optimum range decrease the photosynthetic efficiency of raspberries. Stafne et al., (2001) studied CO_2 assimilation, evapotranspiration and stomata conductance and reported that ‘Nova’ and ‘Reveille’ showed a significant drop in the CO_2 assimilation rate when temperature increased from 20 to 30 °C. It has also been reported that the assimilation rate decreased but evapotranspiration increased in ‘Reveille’ between 30 and 35 °C. Stomatal conductance of ‘Autumn Bliss’ and ‘Reveille’ declined almost 50% when temperature was increased to 30 or 35 °C (Stafne et al., 2001). Leaf gas exchange characters of raspberry cultivars grown at 20 to 35 °C for 2 and 4 weeks were studied and found that ‘Autumn Bliss’
and ‘Reveille’ did not change evapotranspiration but stomatal conductance ($g_s$) declined almost by 50% when temperature increased from 30 to 35 °C (Stafne et al., 2000). Molina-Bravo et al., (2011) reported a lower ratio of variable to maximum chlorophyll fluorescence ($F_v/F_m$) in heat sensitive raspberry cultivars with the lowest values in the afternoon. Diurnal variations in $F_v/F_m$ has also been observed in raspberry and the highest effect of heat stress was reported in the early afternoon of several heat susceptible raspberry cultivars (Molina-Bravo et al., 2011).

In conclusion, evidence suggests that measurement of $F_v/F_m$ can be a good physiological indicator for screening of stress tolerance among cultivars during varietal selection programs and resistance breeding. A low value of $F_v/F_m$ indicates that the light harvesting complex II (PSII) is damaged or inactivated due to production of ROS and reduces carbon fixation during photosynthesis. But the mechanism and reasons for abiotic stress induced decrease in $F_v/F_m$ are complex and there may be errors linked with measurements. To avoid the pitfalls of chlorophyll fluorescence measurement and accuracy, precautions should be taken during design and execution of the experiment along with use of other non-invasive tools.
6.1 Heat sensing mechanism

Plants respond to high temperature stress by altering the composition of certain transcripts, proteins, metabolites and lipids (Mittler et al., 2012). It has also been reported that plant response to high temperature stress involves three mechanisms; 1) sensing of high temperatures, 2) starting signalling pathways, and 3) adjusting metabolism and cell function to protect from heat damages. There is an inward influx of calcium in the plasma membrane which activates signal transduction and the plant acclimatizes to the changes in conditions. If the temperature continues to increase, proteins become unstable in the cytosol and the endoplasmic reticulum. If acclimation is not possible, specific cells or tissues die and leaves, flowers and fruits drop, or even death of the entire plant may occur (programmed cell death) (Ruelland and Zachowski, 2010; Mittler et al., 2012). High temperature stress changes the plant metabolism because proteins, membranes and cytoskeleton structures become unstable and decrease the efficiency of enzymatic activities in the cell (Ruelland and Zachowski, 2010). Moreover, thermal damage occurs primarily at the oxygen evolving complex and inhibits the repair mechanism, cleavage and aggregation of proteins of the photosystem reaction centre (as reviewed by Allakhverdiev et al., 2008).

There is a rapid production and accumulation of ROS at high temperature stress conditions (Mittler, 2002; Almeselmani et al., 2012). ROS is harmful to cellular compounds and decreases cellular metabolic processes. Plants develop strategies to cope with ROS by increasing the expression and activity of ROS scavenging enzymes and the production of antioxidants (Asthir et al., 2009).

In chloroplasts, protein phosphorylation has been reported at high temperature stress (Allakhverdiev et al., 2008). About 20 thylakoid membrane proteins can be phosphorylated. The most important of these phosphoproteins are those of the light harvesting complex II (LHClI) and PSII reaction centre such as D1, D2 and 9-kD polypeptide. The D1 protein is damaged due to the production of ROS which results in reduced CO₂ fixation, O₂ evolution and electron transfer (Allakhverdiev et al., 2008). Phosphorylation of PSII proteins has also been reported to be involved in the stability, degradation, and turnover of the reaction centre.
proteins. Phosphorylation and dephosphorylation of PSII are the main regulatory factors and play a major role in the PSII repair mechanism (Fristedt et al., 2009). There is a positive functional relationship between degradation of D1 protein measured by radioactive labelling and $F_v/F_m$ (Rintamaki et al., 1995). According to Ruelland and Zachowski, (2010) membrane fluidity, protein conformation, cytoskeleton assembly and enzymatic activities are affected by high temperature stress and are interlinked (Figure 6.1). For example, protein conformation could change the enzymatic activities and disassemble the cytoskeleton. The cytoskeleton disassembly may also affect the membrane fluidity. The signalling pathway is down-streamed to further cellular responses such as gene expression, protein accumulation, heat tolerance or cell death in susceptible conditions. The cellular responses to high temperature stress switch off the temperature sensing devices (as reviewed in Ruelland et al., 2009). At the cellular level, there are changes in gene expression profiles, some specific proteins are produced (e.g. heat shock proteins) and there are increases in the heat tolerance capacity in heat tolerant cultivars or programmed cell death occurs in heat susceptible cultivars.

Figure 6.1. Proposed heat sensing mechanism in plants (source: Ruelland and Zachowski, 2010)

Sung et al., (2003) suggested a model and described that high temperature stress provokes two mechanisms; either an increase in cytoplasmic calcium ions or dehydrative stresses inside the cell. It has been reported that the dehydrative stress conveys stress-induced signals to responding genes through respective pathways. These pathways often converge or diverge
from one another. Plants respond to changes in temperature by reprogramming or by altering the composition of certain transcripts, proteins, metabolites and lipids to manage the effects of heat stress on cellular metabolism.

6.2 High temperature stress changes gene expression profiles

As in other stresses, growing plants at high temperature stress triggers defence mechanisms that can be identified as down- or up-regulation in gene expression profiles at the cellular and molecular level (Ahuja et al., 2010). Transcriptomic analysis has shown that the number of heat-stress regulated genes was almost twice that of the recovery regulated genes in grapevine leaves (Liu et al., 2012). The responsive genes identified in the latter study belonged to a large number of important traits and biological pathways including cell rescue (i.e. antioxidant enzymes), proteins (i.e. heat shock proteins), primary and secondary metabolism, transcription factors, signal transduction, and development. Heat stress applied above optimum temperatures in *A. Thaliana* leaves showed strong induction of mRNA for heat shock proteins (Volkov et al., 2003). Changes in the stress related genes are notable at various levels, including the plasma membrane proteins and biochemical pathways operative in the cytosol or cytoplasmic organelles (Sung et al., 2003). The initial effects of heat stress, however, are on plasmalemma, resulting in higher fluidity of the lipid bilayer under stress (Sung et al., 2003).

In field conditions at daytime, high temperature stress coincides with an increase in water loss due to transpiration and cooling effects (Tsukaguchi et al., 2003). Exposure of plants to stress over longer periods alters the leaf water potential and induces changes in the physiological processes of the plant. Increasing temperature results in a decrease in hydraulic resistance mediated by regulation of aquaporin gene expression (Henzler et al., 1999). At the same time, abscisic acid (ABA) is induced which is known to be involved in the survival of plants under drought stress (Maestri et al., 2002). Such stress conditions are likely to become increasingly important for the field production of raspberry in a period of rising temperatures and adoption of tunnel production systems in summer months.

The gene expression of aquaporin is activated during the daytime, when water loss is increased. In *Juglans regia*, a positive relation between the leaf hydraulic conductance and two *PIP*2 aquaporin isoforms (*JrPIP2,1* and *JrPIP2,2*) were observed in response to high
temperature (about 30 °C) and excess light conditions (Cochard et al., 2007). Gene families like aquaporins are water channel proteins capable of transporting water and small molecules across cellular membranes. Three main types of aquaporin 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 transport in relation to environmental conditions (Oliviusson et al., 2001). Tonoplast intrinsic proteins have been shown to act as water channels expressed within storage tissues. Mazzitelli et al., (2007) observed a down-regulation of expressed sequence tags (ESTs) with similarity to an aquaporin gene in raspberry buds during the transition from endodormancy to paradormancy. However, variations exist within crop species in aquaporin isoforms.

In summary, plant cells have interrelated pathways of temperature sensing mechanisms that trigger short-term responses and long-term adaptation. Temperature changes induce cellular responses including membrane fluidity, protein accumulation or depletion, cytoskeleton depolymerization, and metabolic reactions. Heat stress enhances loss of water from plant parts and aquaporin gene families are expressed along with ABA production in response to high temperature stress as in drought stress.
Chapter 7: Factors affecting raspberry fruit quality

7.1 Fruit quality

Fruit quality is defined as the degree of excellence or superiority and the perception of conditions acceptable to consumers (Kader et al., 1985). These perceptions are the attributes which give value to the fruit as evaluated by the consumer. Attributes include edible quality (firmness, sugar acid ratio, aroma, and colour), nutritional quality (minerals, fibres, bioactive compounds, vitamins), export quality (firmness, pest damage, shelf life) etc. Therefore quality always depends on the intended use (e.g., fresh or processed) and covers a range of traits. Berry size, freshness, colour, firmness and shelf-life are considered as physical properties while sweetness, sourness, flavour and nutritional composition are chemical properties (Brennan and Graham, 2009). High yield, good appearance, easy to harvest and long shelf-life for distance shipping are the qualities perceived by the growers while freshness, firmness, size, colour and flavour are qualities considered by most consumers. Vitamins, minerals, dietary fibres and many bioactive compounds are considered to be nutritional qualities (Kader et al, 1985).

7.2 Chemical and nutritional composition of raspberry fruit

Raspberries are popular berry fruits due to a high nutrient content including fibre, essential micronutrients, polyphenols and acids. Raspberry fruits contain 87% water as the main constituent followed by 9% soluble solids and the rest insoluble solids (Table 7.1) (Pritts, 2013). Sugar composes 5 - 6% (Pritts, 2013) and glucose, fructose and sucrose are the main constituents (Wang et al., 2009). The second largest component of the soluble solid is citric acid along with small amounts of malic acid and ten other acids in trace amounts. Raspberries are low in calories (52 kcal 100g⁻¹ fw) and rich in dietary fibre (6.5g 100g⁻¹ fw).

Table 7.1. Range of nutrient content of fresh raspberry fruit (100g) (Source: Pritts, 2013).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount</th>
<th>Minerals</th>
<th>Amount mg</th>
<th>Vitamins</th>
<th>Amount mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (g)</td>
<td>84 - 87</td>
<td>Calcium</td>
<td>22 - 50</td>
<td>Carotene</td>
<td>0.05 - 0.08</td>
</tr>
<tr>
<td>Food energy (kcal)</td>
<td>31 - 49</td>
<td>Iron</td>
<td>0.57 - 1.20</td>
<td>Thiamine</td>
<td>0.01 - 0.03</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>0.42 - 1.40</td>
<td>Magnesium</td>
<td>18 - 30</td>
<td>Riboflavin</td>
<td>0.03 - 0.10</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.20 - 0.55</td>
<td>Phosphorus</td>
<td>12 - 50</td>
<td>Pantothenic acid</td>
<td>0.24 - 0.30</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>5.8 - 11.6</td>
<td>Potassium</td>
<td>130 - 221</td>
<td>Nicotinamide</td>
<td>0.20 - 1.00</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>3.0 - 7.4</td>
<td>Sodium</td>
<td>0 - 2.5</td>
<td>Vitamin B6</td>
<td>0.06 - 0.90</td>
</tr>
<tr>
<td>Ash (g)</td>
<td>0.40 - 0.51</td>
<td>Zinc</td>
<td>0.46</td>
<td>Vitamin C</td>
<td>13 - 38</td>
</tr>
</tbody>
</table>
Manganese 1.01
Sulphur 17.3
Chlorine 22.3 - 22.8
Boron 71 - 125
Copper 0.07 - 0.21

Source: http://www.fruit.cornell.edu/berry/production/pdfs/rasprelfru.pdf

7.3 Polyphenol composition in raspberry fruit

Raspberries are one of the major sources of anthocyanin, catechins, ellagitannins, flavonols, flavones and ascorbic acid (Rao and Snyder, 2010). It has been reported that the common forms of anthocyanidins in raspberry are cyanidin (50%), delphinidin (12%), pelargonidin (12%), malvidin (12%), petunidin (7%), and peonidin (7%). It has been reported that raspberry fruit contains 23 - 59 mg total anthocyanin 100 g⁻¹ fw with cyanidin as a main constituent followed by pelargonidins (Jennings and Carmichael, 1980). The contents of different anthocyanins and polyphenols are presented in Table 7.2 based on ranges reported in the literature.

Table 7.2. Polyphenol composition of raspberry fruit.

<table>
<thead>
<tr>
<th>Components</th>
<th>Cultivar</th>
<th>Amount (mg100 g⁻¹fw)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanin</td>
<td></td>
<td>23 - 59</td>
<td>Jennings and Carmichael, 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>79.2</td>
<td>Beekwilder et al., 2005</td>
</tr>
<tr>
<td></td>
<td>‘Latham’</td>
<td>48.6</td>
<td>Kassim et al., 2009</td>
</tr>
<tr>
<td></td>
<td>‘Glen Moy’</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>‘Glen Ample’</td>
<td>50</td>
<td>Mullen et al., 2002</td>
</tr>
<tr>
<td>Ellagitannin (wild cultivars)</td>
<td></td>
<td>30 - 50% of total phenol</td>
<td>Beekwilder et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.7 - 126.2</td>
<td>Maatta-Riihinen et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>156.0</td>
<td>Maatta-Riihinen et al., 2004</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td></td>
<td>38 - 118</td>
<td>Anttonen and Karjalainen, 2005</td>
</tr>
<tr>
<td></td>
<td>‘Polka’</td>
<td>104 - 114</td>
<td>Ali et al., 2011</td>
</tr>
<tr>
<td>Quercetin-3-glucuronide</td>
<td></td>
<td>1.1</td>
<td>Maatta-Riihinen et al., 2004</td>
</tr>
<tr>
<td>Kaempferol-3-glucuronide</td>
<td></td>
<td>0.6</td>
<td>Maatta-Riihinen et al., 2004</td>
</tr>
<tr>
<td>Pro-anthocyanidin in the form of pro-cyanidin and pro-pelargonidin</td>
<td></td>
<td>78.8</td>
<td>Hellstram et al., 2009</td>
</tr>
</tbody>
</table>

Ellagitannins are the predominant phenolic components with 30-50% of the total phenols in raspberry. The ellagitannin content of raspberries varies among cultivars. De Ancos et al., (2000) analysed Spanish cultivars and found that hydrolysed ellagic acid ranged from 20.7 to
24.4 mg 100 g\(^{-1}\) fw while Anttonen and Karjalainen, (2005) reported that 38-118 mg\(100\ g^{-1}\) fw in Finish cultivars. Maatta-Riihinen et al., (2004) reported that ellagitannin of cultivated raspberries varied from 97.7 - 126.2 mg 100 g\(^{-1}\) fw with the highest (1560 mg\(100\ g^{-1}\) fw) in wild cultivars. The other anthocyanins identified were cyanidin-3-glucorutinoside, pelargonidin-3-sophoroside, pelargonidin-3-glucorutinoside, pelargonidin-3-glucoside, malvidin-3-glucoside, and delphinidin-3-glucoside. Two cultivars, ‘Latham’ and ‘Glen Moy’ contained mid- to low anthocyanin values of 48.6 and 29.7 mg 100 g\(^{-1}\) fw respectively (Kassim et al., 2009). ‘Glen Ample’ contained 50 mg 100 g\(^{-1}\) fw anthocyanins, with cyanidin-3-sophoroside (54%), cyanidin-3-glucorutinoside (23%) and cyanidin-3-glucoside (15%) as main components (Mullen et al., 2002). In addition to anthocyanins, raspberries contain other flavonoids. The primary flavonol glycosides are quercetin-3-glucuronide (1.1 mg\(100\ g^{-1}\) fw), and kaempferol-3-glucuronide (0.6 mg\(100\ g^{-1}\) fw) (Maatta-Riihinen et al., 2004).

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7. 4 Factors affecting raspberry fruit quality and polyphenols

Raspberry fruit quality depends mainly on two factors; cultivar and environment as in many other fruit species (Burrows and Moore, 2002). Although anthocyanin production in
raspberries is genetically regulated, environmental factors also affect it because biosynthesis of anthocyanin is dependent on light exposure (Lister and Lancaster, 1996), temperature (Mori et al., 2005), season and even microclimate within a field or tunnel (Kassim et al., 2009). Some important factors affecting raspberry fruit quality are described below.

7.4.1 Climate
Temperature has significant and contrasting effects on the composition of raspberry bioactive compounds (Remberg et al., 2010). There was higher anthocyanin in bright sunny seasons compared to lower sunlight conditions, and also lower anthocyanin contained in the fruits grown in polytunnels compared to open field conditions due to low light and insufficient UV rays (Kassim et al., 2009). However, the plastics used to cover tunnels often leave out UV rays. It has also been reported that cyanidin-3-glucoside and cyanidin-3-rutinoside were higher in long day conditions. This could probably be an effect of light on anthocyanin biosynthesis. Ultraviolet light induces the accumulation of anthocyanin via stimulating and inducing expression of genes encoding enzymes in the anthocyanin biosynthesis pathways (Guo et al., 2008). Fruits grown in warm and dry summers (day temperature about 25 °C) are sweeter, less acidic, more aromatic and have stronger colour (Prits, 2013). It has also been reported that temperatures greater than 30 °C reduces the aroma of the fruit and wet conditions reduce the sugar content. Jennings and Carmichael, (1980) reported that berries were more aromatic in warm and dry conditions than in mild and humid conditions. The concentration of ascorbic acid and cyanidin-3-rutinoside increased while cyaniding-3-sophoroside decreased with increasing temperature from 12 to 24 °C (Remberg et al., 2010).

The temperature in polytunnels is warmer than in open field conditions but light exposure is reduced which decreases total anthocyanin in raspberries (Kassim et al., 2009). It has also been reported that cyanidin-3-glucoside is the UV-absorbing anthocyanin and therefore is mostly affected by light while pelargonidin glucoside content is not influenced by protected cultivation (Kassim et al., 2009).

7.4.2 Cultivars
Bradish et al., (2011) reported that differences in the flavonoid profile could be due to cultivar differences with covariate effects of temperature and locations. Anttonen et al.,
(2005) also reported that the content of phenolic compounds varied significantly between cultivars. For example, late cultivars had higher anthocyanin than early cultivars (De Ancos et al., 1999). Similarly, anthocyanin is found to be more stable in early cultivars compared to late ripening cultivars (De Ancos et al., 1999; De Ancos et al., 2000).

7.4.3 Organic and conventional field conditions

Organic food is derived from fields where the use of inorganic fertilizers and pesticides have been avoided which helps to promote and increase soil fertility and biological activity, and protect the environment and human health (IFST, 1999; Winter and Davis, 2006). Organic foods are gaining popularity with consumers due to the perception of superior sensory attributes and taste, from the absence of pesticide residues and inorganic fertilisers, and higher levels of nutrients (Williams, 2002; Hargreaves et al., 2008a, b). Many consumers consider organic foods to be more nutritious and are willing to pay an additional 10% to 40% price premium because of the perceived health and nutritional benefits (Winter and Davis, 2006). However, there is no consistency in the reports on differences in quality attributes of organic and conventional foods (Table 7.3) but consistent findings for higher nitrate and lower vitamin C in conventional foods compared to organic foods are reported in the literature (Williams, 2002).

Table 7.3. Examples of inconsistent reports comparing protein, nitrate, vitamins and minerals in organic and conventional grown foods based on a literature review.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Increased with conventional</th>
<th>Same as conventional</th>
<th>Decreased with conventional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein quality</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrate</td>
<td>5</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>21</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>B-vitamins</td>
<td>2</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Fe</td>
<td>15</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>Mg</td>
<td>17</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>Ca</td>
<td>21</td>
<td>20</td>
<td>6</td>
</tr>
</tbody>
</table>

aNumber of studies of organic crops shown to have an increased, decreased or the same nutrient content compared to conventional grown crops (source: Worthington, 1998).

Literature based comparisons have been carried out on the nutritional quality of organically grown products and have reported that organic products contain 27% more vitamin C, 21.1% more iron, 29.3% more magnesium and 13.6% more phosphorus while nitrate levels are
lower from 97% to 819% compared to conventional products (Worthington, 2001; Winter and Davis, 2006). Organic foods also contain higher total sugars and less protein and have a better sensory and long-term storage quality. However, organic products generally have 20% lower yields than conventionally grown products (Rembialkowska, 2007).

Brandt and Molgaard, (2001) explained the factors influencing the levels of compounds in plants with carbon nitrogen (C: N) balance and growth/differentiation balance theories. It has been hypothesized that when nitrogen is abundantly available, plants synthesise nitrogen containing compounds like proteins and secondary metabolites such as alkaloids and glucosinolates. When nitrogen is limited, the metabolism changes more towards carbon containing compounds like starch, cellulose and non-nitrogen-containing secondary metabolites such as phenolics and terpenoids. Similarly, plants always assess the resources available and optimise translocation and distribution towards growth or differentiation.

As with other food commodities, there has been increased interest in consumption of raspberry fruits harvested from organic farms (Graham and Gennings, 2009). The application of chemical pesticides and inorganic fertilizers has been reported to affect the biosynthesis of phenolic compounds in peaches and pears (Carbonaro and Mattera, 2001). It has been reported that all organic peach samples showed a significant increase in polyphenols compared with conventional peaches, while, of the three organic pear samples, two samples were found to have increased polyphenol content compared to the conventionally grown sample. However, reports suggest that total anthocyanins and phenolic compounds and antioxidant activity in fresh raspberries were not consistently affected by the production systems, i.e. organic or conventional, but that total antioxidant activities of fruit differed with farm to farm conditions (Sablani et al., 2010).

### 7.4.4 Maturity stages

The fruit ripening stage also influences anthocyanins, proanthocyanins, ellagitannins and antioxidant compounds (Beekwilder et al., 2005). Polyphenol composition of berry fruit changes throughout the fruit development and ripening stages. When raspberries are green, the tannin content is high and decreases over the ripening period. In contrast, anthocyanins are very low in the green stage, with only cyanidin-3-glucoside present and some traces of cyanidin-3-rutinoside. When fruit are in the pink stage, small quantities of cyanidin-3-
sophoroside and cyanidin-3-glucosylrutinoside are synthesized. When fruits ripen, anthocyanin increases and pelargonidin glycosides begin to synthesize (Beekwilder et al., 2005). When fruits are fully mature, proanthocyanidins continue to decline.

### 7.4.5 Storage and processing

A study was conducted by Kalt et al., (1999) in berries stored at 0, 10, 20, and 30 °C for 8 days and they observed that total phenols and anthocyanin increased by 1.5 and 2.5 fold respectively when stored at 20 °C compared to below or above this temperature. Similarly, Mullen et al., (2002) reported that fruits stored for 3 days at 4 °C contained similar flavonol and individual or total anthocyanin compared to fruits stored for 24 h at 18 °C in ‘Glen Ample’. It has also been found that vitamin C was decreased by 22% and 8% after 3 days of storage and at 4 °C or 1 day of storage at 18 °C, respectively. De Ancos et al., (2000) analysed frozen samples from -20 °C stored for 12 months and found no decrease in total phenolic content while cyanidin-3-glucoside decreased during freezing and storage treatment.

In summary, raspberry fruits are rich in nutrients, anthocyanins and polyphenols. Elagitanin is the major phenol available in the fruit. Berry fruit quality depends primarily on cultivars and environmental factors. Temperature is one of the major factors regulating the quality of the raspberries. Organic and conventional production methods do not consistently affect quality, anthocyanin, phenols and antioxidant capacity but these vary from year to year due to weather conditions and from farm to farm due to niche micro-climates.
8.1 Plant materials

After consideration of the contemporary literature on the effect of elevated temperatures on photosynthetic efficiency, vegetative growth, flowering, fruiting, and fruit yield and quality, we set up one experiment in a climate chamber and thereafter in high tunnel conditions from March to August 2011. A second experiment was established in organic and conventional fields using three annual-and four biannual-fruited raspberry cultivars during August 2010 to September 2013. A list of commercial raspberry cultivars used in this project is presented in Table 8.1.

| Table 8.1. Raspberry cultivars used in experiments during August 2010 to December 2013. |
|-----------------------------------------------|-----------------------------------------------|
| **Experiment I**                             | **Source**                                    |
| Study part I                                  |                                               |
| ‘Autumn Bliss’ (East Mailing, Great Britain),| Commercial nursery-                          |
| ‘Autumn Treasure’ (East Mailing, Great Britain),| Vester Skovgaard,                             |
| ‘Erika’ (East Mailing, Great Britain),        | Denmark                                       |
| ‘Fall Gold’ (East Mailing, Great Britain),    |                                               |
| ‘Polka’ (Poland)                              |                                               |
| **Experiment I**                             | **As in study part I**                        |
| Study Part II                                 |                                               |
| ‘Autumn Bliss’,                               |                                               |
| ‘Autumn Treasure’,                            |                                               |
| ‘Erika’,                                      |                                               |
| ‘Polka’                                       |                                               |
| **Experiment II**                            | **As in study part I**                        |
| Study Part III                                |                                               |
| ‘Autumn Bliss’,                               |                                               |
| ‘Autumn Treasure’,                            |                                               |
| ‘Fall Gold’,                                  |                                               |
| ‘Glen Ample’ (James Hutton Institute, Scotland),|                                               |
| ‘Glen Fyne’ (James Hutton Institute, Scotland),|                                               |
| ‘Octavia’ (East Mailing, Great Britain),      |                                               |
| ‘Tulameen’ (Canada)                           |                                               |
In part I of experiment I, one year old annual-fruiting cultivars were used to evaluate the postulated hypotheses I to IV (Chapter 1). In part II of experiment I, young leaf material was used to analyse the effect of short-term (24 h) heat stress on gene expression profiles in the same cultivars evaluated in the study in part I. In experiment II, field trials were set up in organic and conventional sites of the Research Station, Aarslev, Denmark using one year old plants of seven commercial cultivars (Table 8.1) for yield, sugar and organic acid assessment. Details of materials and methods are explained in Papers I, II and III (Chapter 9 - 11). A brief overview of the experiments specific to particular aspects of research are discussed below.

### 8.2 Study I: Heat stress experiment

#### 8.2.1 Experiment set up

Five annual-fruiting raspberry cultivars were received in the Research Station six weeks after lifting from the nursery Vester Skovgaard, Denmark on January 2011. Plants were stored in a dark cold room (2 ±1°C) for 9, 10 and 11 weeks and therefore the cumulative cold treatment received by the plants was 15, 16 or 17 weeks respectively before potting. The canes were pruned to soil level, potted in 3.5 L pots at 20 - 25 °C and exposed to a photoperiod of 14 h until flower initiation (≈7 weeks after potting).

#### 8.2.2 Heat stress treatment

At the flower initiation stage, plants were transferred into climate chambers at 27, 32 or 37 °C, with a day length of 14 h with constant irradiance of 350 µmol m⁻² s⁻¹ PAR and RH of 60% ±5%. For each temperature, three plants per cultivar and cold store duration were used. The temperature treatment was given for a seven day (168 h). Reference plants were grown under greenhouse at 20 ±5 °C and 14 h light conditions (Figure 8. 1).
20 °C High Tunnel

Daily Fv/Fm measurement

Leaf collection at 24 h heat stress for gene expression study

Five cultivars:

Three cold store duration-15, 16 & 17 weeks
Three replications per cultivar per cold store duration, one plant per replication

Grown in greenhouse:
20 ±5 °C (D/N), 14 h light and 10 h dark periods, 60±5% RH, 350 µmol m⁻²s⁻¹

Flower initiation~7 weeks

20 °C

Greenhouse for 7 days

27 °C

climater chamber for 7 days

32 °C

37 °C

Daily Fv/Fm measurement

Leaf collection at 24 h heat stress for gene expression study

High Tunnel

Evaluation of flowering behavior

Figure 8.1. Heat stress experiment showing the treatment application to five raspberry cultivars.

8.2.3 Evaluation
During the seven days of heat treatment, and the subsequent transfer to tunnels on 24 May 2011 under Danish conditions, the following parameters were evaluated using the specified methods. A brief methodology is illustrated in the Table 8.2.

Table 8.2. A brief methodology used in the evaluation of major parameters of five annual-fruitching raspberries in the study in part I of experiment I.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Materials used</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fv/Fm</td>
<td>MiniPam; Heinz</td>
<td>Daily at 12:00 to 2:00 PM (all cultivars) and daily at 8:30 - 9:00 am, 12:00 - 2:00 PM and 5:30 - 6:30 PM (‘Polka’) on a 30 min dark adapted leaf positioned at third from the top of the main axis</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>Spectrophotometer</td>
<td>At the end of the heat stress treatment (7th day), a</td>
</tr>
</tbody>
</table>
pigments (mg g\(^{-1}\) DW) (UV-1700, Shimadzu, Japan)

leaf positioned at third from the top of the main shoot was sampled. Chemical method was used according to Lichtenthaler, (1987).

Leaf area (m\(^2\))

Leaves developed on the main shoot above the leaf marked on the day of transfer from climate chamber to tunnel conditions after stress period.

First anthesis of terminal flower

First flower opening (king flower) date was recorded and calculated from dormant crown sprouting to opening day.

The percentage of unopened flower buds

\[
\frac{\text{Unopened flower buds}}{\text{Unopened flower buds + flowers and fruit}} \times 100
\]

8.3 Study II- gene expression profiles using microarray and real-time qRT-PCR

8.3.1 RNA extraction

The youngest leaf at the top of the main shoot was collected at 24 h of the plants exposed at 27 and 37 °C and immediately frozen in liquid nitrogen and stored in -80 °C. Total RNA was extracted from the freeze dried leaf (0.1 g) using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's recommendations.

8.3.2 Microarray design and processing

This work was carried out at the James Hutton Institute, Scotland, UK. A *Rubus idaeus* (L.) custom microarray was developed using the Agilent platform from a unigene set assembled from existing sequence resources, comprising transcript sequences isolated from a range of *Rubus* tissues, developmental stages and conditions, including developing fruit and buds. This set was composed of sequences originating from four sources: i) Roche 454 transcripts (52,263); ii) Illumina GAII transcripts (118,275); iii) sanger expressed sequence tags (4,360) and; (iv) bacterial artificial chromosomes (BAC) coding sequences (1,425). In total, 176,833 sequences were assembled using catalyst application profiles (CAP3) software, generating 41,155 contigs and 22,098 singletons. These sequences were BLASTx searched against known plant polypeptide sequences to identify the top protein homologues which, along with
the presence of a polyA or polyT tract, enabled determination of predicted orientation for
55,920 unigenes. Using eArray online software (https://earray.chem.agilent.com/earray/) with
default parameters, a total of 55,708 oligonucleotide probes (one 60mer per unigene) were
designed for generation of a custom Agilent microarray in 8x 60k format (JHI_Ri_60k_v1;
Agilent array design AMADID 035443; ArrayExpress https://www.ebi.ac.uk/arrayexpress accession # A-MEXP-2373.
A simple pairwise microarray experimental plan was devised to utilise the Rubus Agilent 60
k microarray developed at the James Hutton Institute, UK and designed from a unigene set
derived from RNAseq datasets. Agilent Low-Input QuickAMP RNA Labelling Kit was used
to label RNA. Two-Colour Microarray-Based Gene Expression Analysis protocol was used to
hybridize the samples to the Rubus array. Data were reimported into GeneSpring. Comparisons were subsequently made between temperature treatments (27 and 37 °C) for
each cultivar by using volcano plots with threshold of a two-fold change and a Student’s t-
test (two-fold differences in expression level with a P value less than 0.05).

8.3.4 Candidate gene selection and real-time qRT-PCR
Four candidate genes (plasma membrane protein (JHI_Ri_ASM02Jun2011_MMB_8898),
aquaporin (TIP2) (JHI_Ri_ASM02Jun2011_MMB_26875), cysteine protein (JHI_Ri_ASM02Jun2011_MMB_41548) and major latex like protein (JHI_Ri_ASM02Jun2011_MMB_34674)) were selected for further study on the basis of
showing a significant difference in expression levels between cultivars from the array
experiment. However the latter two candidate genes failed to amplify at the required size, and
were therefore discarded in the final real-time qRT-PCR analysis.

Primers/probes were designed for both conventional PCR and real-time qRT-PCR using
either Primer3 (Rozen and Skaletsky, 2000) or the UPL (Universal Probe Library) Assay
Design Centre and recommended parameters from Roche Diagnostics Ltd., UK. In cases
where UPL probes could not be used, the same volumes/concentrations and cycle conditions
were used with the Power SYBR Green PCR Master Mix (Applied Biosystems, UK)
including melt curve analysis to detect any nonspecific amplification. The real-time qRT-
PCR efficiency ($E$) was calculated as suggested by Vaerman et al., (2004).

$$E = 10^{-\frac{1}{S}} - 1;$$ where $E$= efficiency, $S$ = slop values.
8.3.5 Genomic DNA isolation, PCR and sequencing

Genomic DNA was extracted from fresh leaf (1.0 g) by the optimized manual protocol for raspberry for seven cultivars (‘Autumn Bliss’, ‘Autumn Treasure’ ‘Erika’, ‘Glen Fyne’, ‘Octavia’, ‘Polka’ and ‘Tulameen’) for sequencing. Primer combinations were designed using Primer3 (Rozen and Skaletsky, 2000) (Table 8. 3) for PCR.

<table>
<thead>
<tr>
<th>Codes</th>
<th>Cultivar</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB.AqOF-KR</td>
<td>Autumn Bliss</td>
<td>TCCCTCAGGCGATCTTACTTG</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>AB.AqDF_KR</td>
<td>Autumn Bliss</td>
<td>AGTTGCTGGCAACATCTCTGT</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>AB.PMPF-R</td>
<td>Autumn Bliss</td>
<td>TCCGATCCGCAACATTT</td>
<td>CATGCACGTACAAACATA</td>
</tr>
<tr>
<td>AT.pmpOF-R</td>
<td>Autumn</td>
<td>TCCGATCCGCAACATTT</td>
<td>CATGCACGTACAAACATA</td>
</tr>
<tr>
<td>AT.AquaOF-KR</td>
<td>Autumn</td>
<td>TCCCTCAGGCGATCTTACTTG</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Er.AqOF-KR</td>
<td>Erika</td>
<td>TCCCTCAGGCGATCTTACTTG</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Er.AqDF-KR</td>
<td>Erika</td>
<td>AGTTGCTGGCAACATCTCTGT</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Er.PMPF-R</td>
<td>Erika</td>
<td>TCCGATCCGCAACATTT</td>
<td>CATGCACGTACAAACATA</td>
</tr>
<tr>
<td>GF.PMPF-R</td>
<td>Glen Fyne</td>
<td>TCCGATCCGCAACATTT</td>
<td>CATGCACGTACAAACATA</td>
</tr>
<tr>
<td>GF.AqDF-KR</td>
<td>Glen Fyne</td>
<td>AGTTGCTGGCAACATCTCTGT</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Oct.AqOF-KR</td>
<td>Octavia</td>
<td>TCCCTCAGGCGATCTTACTTG</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Pol.AqDF</td>
<td>Polka</td>
<td>TCCCTCAGGCGATCTTACTTG</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Pol.AqOF</td>
<td>Polka</td>
<td>TCCCTCAGGCGATCTTACTTG</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Pol.PMPF-R</td>
<td>Polka</td>
<td>TCCGATCCGCAACATTT</td>
<td>CATGCACGTACAAACATA</td>
</tr>
<tr>
<td>Tul.AqDF-KR</td>
<td>Tulameen</td>
<td>AGTTGCTGGCAACATCTCTGT</td>
<td>TGCGGTTGCAATGACTGTGT</td>
</tr>
<tr>
<td>Tul.PMPF-R</td>
<td>Tulameen</td>
<td>TCCGATCCGCAACATTT</td>
<td>CATGCACGTACAAACATA</td>
</tr>
</tbody>
</table>

Note: Aq- Aquaporin (TIP1), PMP- plasma membrane protein

Single PCR products were treated with ExoSAP-IT (USB® Products Affymetrix, Inc., Ohio, USA) according to the manufacturer’s instructions and sequenced in both directions by Sanger sequencing. Sequences were analyzed and edited manually using Sequencher 4.9 (DNA Codes Corp., Ann Arbor, MI, USA) and aligned using the ClustalW2 multiple sequence alignment programme (www.ebi.ac.uk/Tools/msa/clustalw2/).
8.3.6 Gene mapping

An updated marker map was produced to include both the aquaporin genes (*PIP1* and *TIP2*) using Join Map 3.0 (Van Ooijen and Voorrips, 2001) after PCR amplification and scoring in the 188 individuals of the 'Latham' x 'Glen Moy' mapping population. Map construction was carried out according to the methods described in Graham et al., (2009).

8.4 Study III- Evaluation of raspberry cultivars for fruit yield and berry quality

8.4.1 Experiment set up

Field experiments were established to evaluate seven commercial raspberry cultivars at the Research Centre, Aarslev (10°27’E, 55°18’N), Aarhus University, Denmark in tunnel conditions. One year old plants of three annual- and four biannual-fruited raspberries (Table 8.1) were planted in crop geometry 1.7 x 0.5 m on 19 April 2010 in randomized complete block design with three replications and five plants per replication. An open tunnel of 3.6 m high, 8.5 m wide and 42 m long was built and covered with plastic film. The tunnel was skinned on April 2011, 2012 and 2013 until the end of berry harvesting. Ten canes per meter row were maintained for sampling fruits for yield and quality assessment.

8.4.2 Fertilizer application and plant protection

The organic plot was supplied with organic fertilizer ‘Binadan 5-2-4’ a granular form of fertilizer (5% total N, 1.8% total P and 3.8% water soluble K) (Binadan A/S, Frisbakvej 5, DK-8766, Denmark; [www.binadan.dk](http://www.binadan.dk)) (Table 8.4). Phytoseiulus SD- System (250 mL) was also used to control moths under the tunnel. Two pheromone traps (Agrisense) per tunnel were installed to control raspberry beetle (*Byturus tomentosus*) in April 2012 and plants were sprayed with Spuzit (organic pesticide) (0.1%; dissolved in 1200 L water) on 15 May 2012 and 2013.

Table 8.4. Organic (for basal application) and inorganic (fertigation) schedules in organic and conventional fields.

<table>
<thead>
<tr>
<th>Year</th>
<th>Organic sources</th>
<th>Basal application schedule</th>
<th>Inorganic sources</th>
<th>Fertigation schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>‘Binadan 5-2-4’ (<a href="http://www.binadan.dk">www.binadan.dk</a>)</td>
<td>50% at the end of March, 25% at the beginning of June and 1500 kg ha⁻¹ (176 kg ha⁻¹)</td>
<td>80:20:90 NPK kg ha⁻¹</td>
<td>May to September (2 L m⁻¹ row)</td>
</tr>
</tbody>
</table>
kg N ha\(^{-1}\), i.e. 15 g plant\(^{-1}\)) the remaining 25% in the third week of July

Magnesium was supplied at 15 kg Mg ha\(^{-1}\)

---

2012 ‘Binadan 5-2-4’ 80 kg N ha\(^{-1}\), i.e. 7 g plant\(^{-1}\) 50% at the end of March, 25% at the beginning of June and the remaining 25% in the third week of July

60:20:90 NPK kg ha\(^{-1}\) May to August (2 L m\(^{-1}\) row day\(^{-1}\))

---

2013 ‘Binadan 5-2-4’ ‘Binadan 5-2-4’ was again applied at two times; first at the end of March and second at the beginning of June

60:20:90 NPK kg ha\(^{-1}\) May to July (2 L m\(^{-1}\) row day\(^{-1}\))

---

Dithane (0.2%) and perimor (0.05%; dissolved at 200 L) was also sprayed on 11 June 2012 and 2013.

### 8.4.3 Soil and leaf nutrient analysis

Soil analysis was done before fertilizer application in the fields in 2010. The primary nutrients (nitrate-N, P\(_2\)O\(_5\), K\(_2\)O) and Mg were relatively higher in the conventional field compared to the organic field. In contrast, ammonium-N and humus % were higher in the organic field (Table 8. 5).

#### Table 8. 5. Soil nutrient content of the experimental fields.

<table>
<thead>
<tr>
<th>Reps</th>
<th>Ammonium-N mg/kg</th>
<th>Nitrate-N mg/kg</th>
<th>Humus %</th>
<th>pH(_{CaCl_2})</th>
<th>P(_2)O(_5) g/100</th>
<th>K(_2)O g/100</th>
<th>Mg g/100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.9</td>
<td>1.8</td>
<td>12.2</td>
<td>18.3</td>
<td>3.0</td>
<td>2.7</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>2.4</td>
<td>1.7</td>
<td>10.6</td>
<td>18.8</td>
<td>3.3</td>
<td>2.7</td>
<td>6.1</td>
</tr>
<tr>
<td>3</td>
<td>2.6</td>
<td>1.6</td>
<td>7.8</td>
<td>17.5</td>
<td>2.9</td>
<td>2.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Mean</td>
<td>2.6</td>
<td>1.7</td>
<td>10.2</td>
<td>18.2</td>
<td>3.1</td>
<td>2.6</td>
<td>6.4</td>
</tr>
</tbody>
</table>
O, organic; C, conventional

Plant leaf tissues were also analysed to assess the nutrient contents of leaf after harvesting in 2012. Nitrogen and magnesium content were higher in the organic field in ‘Autumn Bliss’ and ‘Tulameen’ while potassium was higher in ‘Tulameen’ in the conventional field. There were no differences in macronutrients and micronutrients of leaf tissues between cultivars from the organic and conventional fields.

8.4.4 Fruit sampling for yield and berry quality evaluation

Fruits were picked twice a week from week 28 to 36 of each season for the biannual- and week 32 to 44 for annual-fruiting cultivars (Table 8.6). Berries were harvested from a one meter row containing 10 canes at the full ripe stage. The total marketable fruit yield and the weight of 20 representative fruit weight were recorded for the calculation of fruit size.

Table 8.6. Fruit sampling schedules for yield, size and quality analysis.

<table>
<thead>
<tr>
<th>Year</th>
<th>Annual-fruiting cultivars</th>
<th>Biannual-fruiting cultivars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield and berry size</td>
<td>Total soluble solid and organic acids</td>
</tr>
<tr>
<td>2011</td>
<td>week no. 32 to 44</td>
<td>week no. 36 and 37</td>
</tr>
<tr>
<td>2012</td>
<td>week no. 32 to 44</td>
<td>week no. 36 and 38</td>
</tr>
<tr>
<td>2013</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fruit samples were stored at -20 °C for sugar and organic acid analysis in 2011 and 2012. Total soluble solid (TSS, °Brix) was measured using a digital refractometer (Pocket PAL-1, Atago, Japan). Titratable acid (TA) was analysed using a digital biuret and 0.1 M NaOH to
titrate samples to an endpoint of 8.1, and expressed based on the percentage of citric acid equivalents.

Fifty grams of fresh berries collected in the 3rd week from the start of harvesting was stored at -20 °C for sugar and organic acid analysis. After being fridge dried, the samples were ground into fine powders, vacuum packed and stored in a fridge before analysis. Two major organic acids (citrate and malate) were quantified following extract dilution (1:20) by anion exchange HPLC on a Dionex IonPac AS11-HC 4 x 250 mm column fitted with a 4 x 50 mm guard column according to a method based on that of Nwanko et al., (2012). A detail of the protocol has been explained in Chapter 11 (Manuscript III).

Three major sugars (glucose, fructose and sucrose) were quantified following extract dilution (2: 1000) by anion exchange chromatography on a Dionex Carbopac PA-100 250 x 4 mm column. The mobile phase was 200 mM NaOH prepared in degassed water pumped at 1 mL per min for 15 min in isocratic mode. Sugars were detected by pulsed amperometry using a standard quad waveform and quantified by reference to a standard curve for each of sucrose, glucose and fructose.

### 8.4.9 Weather data collection

During the experimental period from 2010 to 2013, temperature, light intensity, relative humidity, precipitation and wind speed inside and outside the tunnels were recorded daily at weather stations within the Research Centre, Aarslev, Denmark. The details of the temperature data were used to investigate the effect of one week pre-harvest temperature on yield and quality of commercial raspberries in Danish conditions. The details of the temperature inside and outside the tunnels by year has been illustrated in Chapter 11 (Manuscript III, Figure 1).
Chapter 9: Paper I

Chlorophyll Fluorescence and Flowering Behaviour of Annual-Fruiting Raspberry Cultivars under Elevated Temperature Regimes

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Chlorophyll Fluorescence and Flowering Behaviour of Annual-Fruiting Raspberry Cultivars under Elevated Temperature Regimes

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(¹Department of Food Science, Aarhus University, Denmark, ²GartneriRådgivningen A/S, HortiAdvice Scandinavia, Denmark and ³The James Hutton Institute, Scotland, UK)

Summary

The effects of a seven day period with increased temperature from a standard level of 20 to 27, 32 or 37 °C during flower initiation on apparent quantum efficiency (Fv/Fm), chlorophyll content and flowering behaviour in raspberry 'Autumn Bliss', 'Autumn Treasure', 'Erika', 'Fall Gold' and 'Polka' were investigated. The Fv/Fm decreased steadily from 0.82 to 0.70 on the seventh day at 37 °C in 'Autumn Bliss' and 'Fall Gold'. A significant diurnal variation in Fv/Fm, characterized by a midday depression and partial recovery in the evening was observed in 'Polka'. Plants of 'Autumn Bliss' exposed for seven days to 37 °C had 58 % less chlorophyll a as compared to those grown at 20 °C. The chlorophyll a/b ratio decreased to 20 % at 37 °C in 'Autumn Bliss', and 'Fall Gold'. The number of days to anthesis of the terminal flower was not significantly affected by the temperature treatments. The number of unopened axillary buds decreased at 37 °C in 'Autumn Bliss', 'Autumn Treasure', 'Fall Gold' and 'Polka'. The percentage of flowering lateral shoots per plant decreased by 16 % at 37 °C in 'Autumn Bliss' whereas it increased by 7 % at 37 °C in 'Autumn Treasure' and 'Erika'. Seven days at 37 °C during flower induction delayed flowering in 'Autumn Treasure' and 'Erika' resulting in 20 % less unopened flowers at the time of registration compared with reference plants. Thus the negative responses of heat stress was reflected in a decreased midday Fv/Fm in all cultivars, while there was a remarkable difference in chlorophyll content and flowering behaviour among cultivars. These responses suggest that there is a difference of annual-fruiting raspberry cultivars in their inherent ability to adapt to heat stress.

Key words. chlorophyll content – photosynthesis – Rubus idaeus – heat stress – terminal flower

Introduction

Raspberry (Rubus idaeus L.) is an important soft fruit crop across cold and temperate regions of the world (Heide and Sonstebry 2011; Sonstebry and Heide 2012) and interest in raspberry production under open-field, high-tunnel and greenhouse conditions has been increasing (Oliveira et al. 2002; Dale et al. 2003, 2005). Manipulation of the growth cycle in annual-fruiting raspberry cultivars allows a year-round production of fruit in the greenhouse (Dale et al. 2005). However, the effect of temperature on flower formation in annual-fruiting cultivars is being discussed (Heide and Sonstebry 2011). The optimum temperature for annual-fruiting raspberry cultivation ranges from 16 to 24 °C, but some cultivars like 'Polka' grow well even at 30 °C (Heide and Sonstebry 2011). In annual-fruiting raspberries, the environmental regulation of flower induction is not fully understood (Carew et al. 2001; Sonstebry and Heide 2010). But flowering is advanced by intermediate photoperiods (11–15 h) and temperatures between 20 and 25 °C (Carew et al. 2001; Sonstebry and Heide 2010; Neri et al. 2012). Most raspberry cultivars are poorly adapted to warm and humid conditions that may occur during summer in temperate zones (Ballington and Fernandez 2008).

Temperatures above optimum negatively affects plant growth in all developmental stages from shoot formation in the spring to flowering and fruit ripening. However, the upper temperature threshold for optimal growth varies significantly at different phenological and growth stages. Excessively high temperature (10–15 °C above optimum) adversely affects photosynthesis, respiration, evapotranspiration, membrane integrity and modulates hormone and metabolite production (Wahid et al. 2007). Photosynthesis is one of the most heat-sensitive processes in plants (Berry and Bjorkman 1980; Wahid et al. 2007) and many authors have shown that elevated temperature reduces net assimilation due to impairment of CO₂ fixa-
tion, photosphosphorylation and the electron transport chain (SALVUCCI and CRAFTS-BRANDNER 2004a, b). Furthermore, high temperature decreases photosynthetic efficiency, chlorophyll accumulation and regulates proteins (EFEOGLU and TERZIOGLU 2009). MOLINA-BRAVO et al. (2011) reported a lower ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) in heat sensitive raspberry cultivars with the lowest values in the afternoon. Moreover, heat stress may decrease the total concentration of chlorophyll pigments, change the ratio of chlorophyll a to b (Chl a/b), and chlorophyll to carotenoid content (a+b/x+c) in stressed leaves depending on the temperature tolerance of the species (CAMEJO et al. 2005; EFEOGLU and TERZIOGLU 2009).

An increasing interest in producing raspberries in warmer climates and out-of-season in protected cultivation has stimulated research aimed at a better understanding of the effects of temperature and photoperiod on growth and fruiting (CAREW et al. 2000, 2001; DALE et al. 2003; SONSTEBY and HEIDE 2010). In this study, the effects of high temperature during early flower initiation on flowering behaviour of five annual-fruiting raspberry cultivars were investigated to pin point possible control mechanisms underlying the differences between cultivars. The aim of the study was also to determine if chlorophyll fluorescence may be used as a screening criterion for high temperature sensitivity in annual-fruiting raspberry cultivars.

Materials and Methods

Plant material and experimental conditions

One-year old cold-stored plants of five annual-fruiting raspberry cultivars; 'Autumn Bliss', 'Autumn Treasure', 'Fall Gold', 'Erika' and 'Polka' were obtained from the nursery Vester Skovgaard, Denmark, where they were lifted from the field in mid-November 2010 and kept in a dark cold-storage at 2 °C before shipping to the Department of Food Science, Aarhus University on 20 January 2011. The plants were stored on site in a dark cold room (2 ± 1 °C) for additional 9, 10 or 11 weeks, which resulted in cold-storage for a total of 15, 16 or 17 weeks before forcing under greenhouse conditions. The canes were pruned to soil level and potted in 3.5 L pots containing 10–30 mm blonde peat substrate (Pindstrup No 4, Pindstrup Mosebrug A/S, Ryomgard, Denmark), with an electrical conductivity (EC) of 2–4 mS cm⁻¹ and pH 6. Plants were forced at 20–25 °C and a photoperiod of 14 h until flower initiation. Microscopic observation of axillary buds from non-experimental plants was carried out five weeks after root sprouting to determine the time of flower initiation. Flower primordia were visible under the microscope after seven weeks (WILLIAMS 1959). A single primary shoot was maintained per pot during the experimental period. Plants were fertigated once a day to pot capacity using a nutrient solution with an EC of 2.16 mS cm⁻¹ containing 40 mg L⁻¹ NH₄-N, 165 mg L⁻¹ NO₃-N, 44 mg L⁻¹ P and 257 mg L⁻¹ K.

Heat stress treatments

When plants reached the stage of flower initiation (~seven weeks after root sprouting), they were transferred to three climate chambers (MB-teknik, Broendby, Denmark) at 27, 32 or 37 °C, with a day length of 14 h similar to the greenhouse. There were three plants per cultivar, treatment and cold-storage time (15, 16 and 17 weeks). The temperature treatment was given for a seven day (~168 h) period. A fourth set of plants remained in the greenhouse at a temperature ranging from 20 to 25 °C and 14 h light conditions as reference. In the climate chambers, the irradiance was constant at 350 μmol m⁻² s⁻¹ PAR and RH was 60 ± 5 % for all replications. All plants were fertigated with a complete nutrient solution to pot capacity daily at 08.00 am and 05.00 pm.

Measurement of chlorophyll fluorescence

During the seven days of heat treatment, chlorophyll fluorescence was measured on the third leaf (~75 % expanded) from the top of the shoot between 12.00 am to 02.00 pm each day to minimize the diurnal effects of temperature and light on photosynthesis. A pulse-amplitude modulated fluorometer (MiniPam; Heinz Walz GmbH, Effeltrich, Germany) was used on the upper surface after 30 min dark adaptation using a standard leaf clip. Initial fluorescence (F₀), when plastoquinone electron acceptor pool (QA) is fully oxidized and maximum fluorescence (Fm), when QA is transiently fully reduced, were recorded for photosystem II and variable fluorescence (Fv = Fm − F₀) and maximum quantum efficiency (Fv/Fm) were calculated. Readings of Fv/Fm were taken on the third leaf (~75 % expanded) from the top of the shoot in ‘Polka’ between 08.30 and 09.00 am, 12.00 am and 02.00 pm, and 05.30 and 06.30 pm to describe diurnal variations in apparent quantum efficiency.

Chlorophyll pigment

At the end of the heat stress treatment, half of each leaf used for Fv/Fm measurements was collected and weighed and immediately frozen in liquid nitrogen and stored at ~80 °C. The samples were freeze-dried for 48 h and homogenised into a fine powder. Approx. five mg of the homogenised freeze-dried tissue was weighed in a 15 mL test-tube and 100 μL distilled water was added. After 10 min of hydration, 8 mL of 96 % ethanol was added before shaking in a vortex at approx. 250 rpm for 1 min. The samples were wrapped in aluminium foil and incubated at room temperature in an exhaust hood overnight. Samples were vortexed for one min and the absorbance was measured on the supernatant at 470.0, 648.6, 664.2
and 750.0 nm using spectrophotometer (UV-1700, Shimadzu, Japan). The chlorophyll a, chlorophyll b, chlorophyll a/b and the ratio of chlorophyll (a + b) to carotenoid (x + c) were calculated as described by LICHTENTHALER (1987).

Plant growth and flowering behaviour

After the seven day treatment at elevated temperature regimes in climate chambers, plants were transferred to an open high tunnel (Atoplan Longlife film, Vorden, The Netherlands), and the third node from top of the shoot was marked to indicate new shoot and leaf development after stress treatment. The plants were placed in rows on a black ground cover (Mypex) with an inter-row spacing of 1.0 m and 0.3 m between plants. Plants were drip-irrigated with a complete nutrient solution. For each plant, the day of anthesis of the terminal flower was recorded and plant growth and flowering behaviour were evaluated 30 days after anthesis of the terminal flower. The number of days to anthesis of the terminal flower was counted from the day of root sprouting when forcing them in greenhouse. The following were recorded: leaf area (LI-3100 Leaf Area Meter, LI-COR, Lincoln, USA) of leaves developed above the marked node (main shoot developed after the stress period), total number of lateral shoots, number of flowering lateral shoots and number of unopened axillary buds on the main shoot. The percentage of unopened flower buds was calculated as: Unopened flower buds (%) = (Unopened flower buds) * 100/(Unopened flower buds + flowers and fruit)

Experimental design and statistical analysis

The experiment was a split plot design with three cold-storage duration as main-plot and temperature as sub-plot. Repeated (Fv/Fm) measurements were conducted every day during the seven day temperature treatment. Statistical analysis was carried out using the SAS procedure PROC MIXED (SAS Inst. Inc., Cary, NC). Each cultivar was analysed separately. Data were tested for normal distribution and homogeneity of variance before analysis. The percentage and proportional data were arcsine transformed prior to statistical analysis, original mean values are shown in figures and tables. Mean differences within cultivar, temperature and cold storage duration were separated using Tukey Kramer’s test at P < 0.05.

Results

Chlorophyll fluorescence

Three-way interaction between cold-storage duration, temperature and days of temperature stress was found for all cultivars (Table 1 and Fig. 1). The Fv/Fm decreased with increased stress period at 27, 32 and 37 °C and the highest reduction was from 0.82 to 0.70 at 37 °C at day seven in ‘Autumn Bliss’ and ‘Fall Gold’. While in ‘Autumn Treasure’ and ‘Erika’, the Fv/Fm was reduced to 0.72 at day seven at 37 °C. A similar pattern for Fv/Fm during the period of increased temperature was found for plants cold-stored for 15 and 16 weeks but for plants cold stored for 17 weeks, Fv/Fm dropped more quickly between days one and five. There was an abrupt drop in Fv/Fm at 32 and 37 °C in all cultivars until day five of stress treatment.

Diurnal variations in Fv/Fm in ‘Polka’

Similar to the other four cultivars, midday Fv/Fm in ‘Polka’ decreased from 0.82 to 0.71 at the end of the seven day stress period (Fig. 2). However, the Fv/Fm ratio remained constantly higher in the morning and evening as compared with midday (P < 0.001). When statistical analysis was carried out using cold-storage duration (C), temperature (T), days of temperature stress (S), and time of day (D) as class variables, the four-way (C*T*S*D) and

Table 1. Main effects and interactions of cold-storage duration (C), temperature (T) and days of temperature stress (S) on quantum efficiency (Fv/Fm) in five annual-fruitting raspberry cultivars using PROC MIXED with stress period as repeated variable.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Df</th>
<th>‘Autumn Bliss’</th>
<th>‘Autumn Treasure’</th>
<th>‘Erika’</th>
<th>‘Fall Gold’</th>
<th>‘Polka’</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0030</td>
</tr>
<tr>
<td>T</td>
<td>3</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C*T</td>
<td>6</td>
<td>0.0310</td>
<td>0.0002</td>
<td>&lt; 0.0001</td>
<td>0.0030</td>
<td>ns</td>
</tr>
<tr>
<td>S</td>
<td>7</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
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<td>0.0050</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>ns</td>
</tr>
<tr>
<td>T*S</td>
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<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C<em>T</em>S</td>
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<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0070</td>
<td>&lt; 0.0001</td>
<td>0.0140</td>
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</tbody>
</table>

Df: degree of freedom; ns: not-significant at P > 0.05

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three-way (C*T*D) interactions were not significant while C*T*S and T*S*D were significant for \( F_v/F_m \) (\( P < 0.05 \)).

**Chlorophyll pigments**

The effect of temperature on chlorophyll pigments varied with cultivar. The Chl a and Chl a/b significantly decreased with increasing temperature in ‘Autumn Bliss’, ‘Fall Gold’ and ‘Polka’, while there was no effect in ‘Autumn Treasure’ and ‘Erika’ (Fig. 3A). The decrease in Chl a at 37 °C ranged from 20 to 58 % in ‘Autumn Bliss’ and ‘Fall Gold’, respectively, when compared with greenhouse conditions and resulted in yellowing of the upper leaves. Similarly, Chl a/b decreased from 5 to 20 % in ‘Autumn Bliss’, ‘Fall Gold’ and ‘Polka’ at 37 °C (Fig. 3B). The chlorophyll to carotenoid \((a+b)/(x+c)\) ratio significantly decreased in ‘Erika’, with increased temperature (Fig. 3C). Therefore ‘Autumn Treasure’ and ‘Erika’ could not be regarded as heat sensitive according to these parameters.

**Fig. 1.** The effect of heat stress treatment (days) and preceding cold-storage duration (15, 16 or 17 weeks) on \( F_v/F_m \) of five annual-fruiting raspberry cultivars during a seven day period. On day 0, \( F_v/F_m \) was measured in the greenhouse before transfer to climate chamber (\( n = 3 \)). Vertical bars indicate standard error of mean (\( n = 3 \)).
Growth and flowering

The leaf area of the main shoot developed between a seven day period at 37 °C and 30 days after anthesis of the terminal flower was significantly lower (P < 0.05) in ‘Autumn Treasure’, ‘Erika’, ‘Fall Gold’, and ‘Polka’ and the reduction ranged from 68 to 82 % compared with reference plants grown in greenhouse. The number of unopened axillary buds at the main shoot decreased significantly after seven days at 37 °C in ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Fall Gold’ and ‘Polka’ whereas the number of lateral shoots per plant was increased in ‘Autumn Bliss’ (175 %), ‘Erika’ (66 %) and ‘Fall Gold’ (31 %) compared with reference plants (Table 2). The percentage of flowering lateral shoots per plant decreased by 16 % in ‘Autumn Bliss’ after exposure to 37 °C but other cultivars were not affected by the temperature treatments. The height of the main shoot following a seven day stress period at 37 °C was significantly reduced in ‘Autumn Treasure’, ‘Erika’ and ‘Polka’. The number of days to anthesis of the terminal flower was not influenced by increased temperature in any of the five cultivars (Fig. 4) but the percentage of unopened flower buds was decreased up to 22 % in ‘Autumn Treasure’ and ‘Erika’ by a seven day period at 37 °C compared to greenhouse conditions. However, the number of ripe fruits at 30 days after anthesis of the terminal flower was not affected by increased temperatures during flower initiation (data not shown).

The leaf area of the main shoot, developed after heat stress, increased significantly in ‘Autumn Bliss’ and ‘Erika’

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with increased cold-storage period (Table 3). The number of unopened axillary buds was higher and the number of lateral shoots per plant lower when the storage period was extended in ‘Autumn Bliss’ and ‘Fall Gold’, but was not affected in the remaining three cultivars. Also a higher number of flower buds per lateral was found in ‘Autumn Bliss’ (72 %) and ‘Fall Gold’ (29 %) after 17 weeks of cold storage compared with 15 weeks, whereas the percentage of flowering lateral shoots was only influenced in ‘Autumn Bliss’. Plants of ‘Autumn Bliss’, ‘Erika’ and ‘Fall Gold’ were higher after 17 weeks of cold storage compared with 15 and 16 weeks.

### Discussion

**Chlorophyll fluorescence**

The effect of extreme temperatures on plant growth, development and reproductive behaviour is complex due to the combined effect of environment and genetic factors. The damage caused by high temperatures includes a wide range of structural and functional changes in plants (Georgieva et al. 2000). At temperatures above the optimum, the apparent quantum yield declines due to inhibition of PSII activity (Berry and Bjorkman 1980). The heat

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**Table 2. Effect of a seven day period of elevated temperature during flower initiation on growth and flowering behaviour in five annual-fruiting raspberry cultivars.**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Leaf area (cm²) †</th>
<th>Unopened axillary buds plant⁻¹ ¥</th>
<th>No of lateral shoots plant⁻¹ ¥</th>
<th>Flowering lateral shoots plant⁻¹ (%) ¥</th>
<th>No of flower and buds lateral⁻¹ ¥</th>
<th>Main shoot height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Autumn Bliss’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gh (20 °C)</td>
<td>1948⁻ᵃ</td>
<td>8⁻ᵇ</td>
<td>7⁻ᶜ</td>
<td>95.0⁻ᵃᵇ</td>
<td>35⁻ᵃ</td>
<td>145⁻ᵃ</td>
</tr>
<tr>
<td>27 °C</td>
<td>1717⁻ᵃ</td>
<td>10⁻ᵃ</td>
<td>6⁻ᶜ</td>
<td>96.8⁻ᵃ</td>
<td>37⁻ᵃ</td>
<td>145⁻ᵃ</td>
</tr>
<tr>
<td>32 °C</td>
<td>1469⁻ᵃ</td>
<td>7⁻ᵇ</td>
<td>9⁻ᵇ</td>
<td>92.7⁻ᵃᵇ</td>
<td>31⁻ᵃ</td>
<td>136⁻ᵃ</td>
</tr>
<tr>
<td>37 °C</td>
<td>1185⁻ᵃ</td>
<td>5⁻ᶜ</td>
<td>12⁻ᵃ</td>
<td>85.7⁻ᵇ</td>
<td>29⁻ᵃ</td>
<td>131⁻ᵃ</td>
</tr>
<tr>
<td>‘Autumn Treasure’</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gh (20 °C)</td>
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<td>5⁻ᵃᵇ</td>
<td>10⁻ᵃ</td>
<td>96.0⁻ᵃ</td>
<td>42⁻ᵃ</td>
<td>189⁻ᵃ</td>
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<tr>
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<td>2741⁻ᵃᵇ</td>
<td>7⁻ᵃ</td>
<td>11⁻ᵃ</td>
<td>97.1⁻ᵃ</td>
<td>49⁻ᵃ</td>
<td>178⁻ᵃ</td>
</tr>
<tr>
<td>32 °C</td>
<td>3566⁻ᵃ</td>
<td>9⁻ᵃ</td>
<td>10⁻ᵃ</td>
<td>99.0⁻ᵃ</td>
<td>54⁻ᵃ</td>
<td>178⁻ᵃ</td>
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<tr>
<td>37 °C</td>
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<td>3⁻ᵇ</td>
<td>15⁻ᵃ</td>
<td>100.0⁻ᵃ</td>
<td>42⁻ᵃ</td>
<td>138⁻ᵃ</td>
</tr>
<tr>
<td>‘Erika’</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gh (20 °C)</td>
<td>9455⁻ᵃ</td>
<td>8⁻ᵃ</td>
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<td>9⁻ᵃᵇ</td>
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<td>9⁻ᵃ</td>
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<td>56⁻ᵃ</td>
<td>173⁻ᵇ</td>
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<tr>
<td>‘Fall Gold’</td>
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<td></td>
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<tr>
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<td>27⁻ᵃ</td>
<td>130⁻ᵃᵇ</td>
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<tr>
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<td>9⁻ᵃ</td>
<td>8⁻ᵇ</td>
<td>88.7⁻ᵃ</td>
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<tr>
<td>37 °C</td>
<td>862⁻ᵇ</td>
<td>5⁻ᵇ</td>
<td>11⁻ᵃ</td>
<td>91.4⁻ᵃ</td>
<td>28⁻ᵃ</td>
<td>120⁻ᵇ</td>
</tr>
<tr>
<td>‘Polka’</td>
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</tr>
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<td>7⁻ᵃ</td>
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<td>52⁻ᵃ</td>
<td>163⁻ᵃ</td>
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<td>3065⁻ᵃᵇ</td>
<td>9⁻ᵃ</td>
<td>8⁻ᵃ</td>
<td>98.8⁻ᵃ</td>
<td>53⁻ᵃ</td>
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<td>3323⁻ᵃᵇ</td>
<td>9⁻ᵃ</td>
<td>6⁻ᵃ</td>
<td>92.3⁻ᵃ</td>
<td>55⁻ᵃ</td>
<td>148⁻ᵃᵇ</td>
</tr>
<tr>
<td>37 °C</td>
<td>974⁻ᵇ</td>
<td>5⁻ᵇ</td>
<td>9⁻ᵃ</td>
<td>98.3⁻ᵃ</td>
<td>51⁻ᵃ</td>
<td>117⁻ᵇ</td>
</tr>
</tbody>
</table>

†: Leaf area of the leaves developed after the temperature stress period

¥: Data were log transferred prior to statistical analysis but original mean values are shown

Gh: Greenhouse

Different letters within the same column and cultivar indicate significant difference at P < 0.05 by Tukey-Kramer test
stress has a direct effect on the PSII photo-oxidizing site and decreases the emission of the variable chlorophyll fluorescence (Georgieva et al. 2000). The dark-adapted value of $F_v/F_m$ is therefore a sensitive indicator of maximal photosynthetic performance with optimal values around 0.83 for most plant species ( Bjorkman and Demming 1987). In our observations, midday $F_v/F_m$ decreased steadily from 0.82 to 0.70 at 37 °C over a 168 h exposure period. This decrease in $F_v/F_m$ was only a weak and recoverable effect and therefore not likely to have any major impact on the D1 protein repair system ( Aro et al. 1993; Leipner 2007; Allakhverdiev et al. 2008). In our experiment, $F_v/F_m$ for ‘Polka’ was always higher in the morning and evening than at midday regardless of temperature treatment and period of cold-storage. The midday $F_v/F_m$ decreased with increased stress period and temperature ($P < 0.001$). The depression at midday and the partial recovery in the evening indicated that photoinhibition was reversible in ‘Polka’. Moreover, it was observed that $F_v/F_m$ did not fully recover in the morning after consecutive stress periods thus the repair mechanism was not sufficient. Diurnal variation in $F_v/F_m$ has previously been observed in raspberry, but in contrast to our results, the highest effect of heat stress was observed in the early afternoon of several heat susceptible raspberry cultivars ( Molina-Brazo et al. 2011). But similar to the present study, $F_v/F_m$ was fully or partly recovered by the end of the photoperiod in soybean and Heteromeles even after a severe drop at midday due to direct sun exposure ( Kao and Forsyth 1992; Valladares and Pearcy 1997). Sharma et al. (2012) also observed a diurnal variation in $F_v/F_m$, comparatively higher in morning than in afternoon and evening in greenhouse-grown wheat. The significantly stronger depression of $F_v/F_m$ in plants cold-stored for 17 weeks, as compared to 15 and 16 weeks, may imply the operation of a quantitative chilling effect, but this could not be validated by the present study. The $F_v/F_m$ measurement indicates that all five cultivars have an almost similar response with regard to heat tolerance.

**Chlorophyll pigments**

Elevated temperature regimes affect the total concentration of chlorophyll pigments in leaves depending on the thermotolerance capacity of the species ( Camacho et al. 2005; Guo et al. 2006; EfEOGLU and TErZIOGLU 2009). The Chl a/b ratio is an indicator of the functional pigment equipment and light adaptation/acclimation capacity of the photosynthetic apparatus. Chl b is found exclusively in the pigment antenna system, whereas Chl a is present in the reaction centres of PSI and PSII as well as in the pigment antenna ( Guo et al. 2006). We observed that the Chl a and Chl a/b decreased significantly at high temperature in ‘Autumn Bliss’ and ‘Fall Gold’ compared to greenhouse conditions. The low Chl a/b suggests a decrease in the ratio of reaction centres compared to light harvesting proteins while the lower Chl a content suggests a decrease in light harvesting capacity ( Adams and Barker 1998). The chlorophyll concentration decreased in ‘Sutsuma’ mandarin, when the temperature was increased to 38 °C for a 15-days stress period ( Guo et al. 2006). The measured levels of Chl a and Chl a/b indicate that ‘Autumn Bliss’ and ‘Fall Gold’ are less heat tolerant than ‘Autumn Treasure’ and ‘Erika’ that are not heat sensitive according to these parameters.

**Growth and flowering**

Due to their temperate origin, the plasticity of current raspberry cultivars to adapt to high temperature is limited ( Ballington and Fernandez 2008). Increased temperature up to 24 °C advanced anthesis and increased number of leaves in ‘Autumn Bliss’ ( Carew et al. 2003; Heide and Sonsteby 2011). In our study, the opening date of the terminal flower was not significantly affected by heat stress during flower initiation, presumably because stress was imposed for a short period. However, at high temperature
(37 °C), anthesis of the terminal flower in 'Autumn Bliss', 'Fall Gold' and 'Polka' was earlier, while in 'Autumn Treasure' and 'Erika', it tended to be delayed. SONSTEBY and HEIDE (2010) observed that flowering and fruit maturation was advanced by elevated temperature from 20 to 26 °C in 'Autumn Bliss' but delayed in 'Autumn Treasure' above 20 °C. There was a higher number of lateral shoots in 'Autumn Bliss' in the shorter cold-storage period. The result is surprising because the chilling periods used were very much longer (> 15 weeks) than those suggested by others to satisfy the chilling requirement (TAKEDA 1993; CAREW et al. 2001). Chilling is not an absolute requirement of annual-fruiting raspberries but cold treatment advances the day-to-flower opening in many annual cultivars (TAKEDA 1993; HEIDE and SONSTEBY 2011). It has been shown that flowering was advanced when chilling duration increased from 0 to 10 weeks in 'Autumn Bliss' (CAREW et al. 2001). As chilling duration increased, the rate of vegetative growth increased and days-to-first flower opening decreased in 'Autumn Bliss'. Similarly, cold treatment affected flower bud development. For example, non-chilled 'Heritage' plants developed 15 flowering lateral shoots, while plants receiving > 750 chilling units had 25 flowering lateral shoots (TAKEDA 1993). Therefore low temperature exposure, also known as vernalization prior to shoot growth is needed for flower bud initiation (TAKEDA 1993; HEIDE and SONSTEBY 2011). Our results are in agreement with CAREW et al. (2001), who reported that cold-storage influences vegetative and flowering behaviour of raspberry cultivars. The differences could also be due to the loss of carbohydrates during cold-storage and differences in climate conditions in the greenhouse, although the temperature was maintained similar for each replication, light and RH obviously were not fully controlled in greenhouse as in the climate chambers.

**Conclusion**

Short exposure of annual-fruiting raspberry cultivars to high temperature decreases midday $F_{m}/F_{m}$ and in some

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Table 3. Effect of cold-storage period on growth and flowering behaviour in five annual-fruiting raspberry cultivars.

<table>
<thead>
<tr>
<th>Cold-storage period</th>
<th>Leaf area $(cm^{-2})^\dagger$</th>
<th>Unopened axillary buds plant$^{-1}^\text{Y}$</th>
<th>No of lateral shoots plant$^{-1}^\text{Y}$</th>
<th>Flowering lateral shoots plant$^{-1}^\text{Y}$</th>
<th>Number of flowers and buds lateral$^{-1}^\text{Y}$</th>
<th>Main shoot height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Autumn Bliss'</td>
<td>1171 $^b$</td>
<td>5 $^b$</td>
<td>10 $^a$</td>
<td>87.1 $^b$</td>
<td>25 $^b$</td>
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<tr>
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<td>1274 $^{ab}$</td>
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<td>7 $^b$</td>
<td>91.4 $^{ab}$</td>
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<tr>
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<tr>
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<td>99.3 $^a$</td>
<td>48 $^a$</td>
<td>170 $^a$</td>
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<td>5 $^b$</td>
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<td>98.0 $^a$</td>
<td>72 $^a$</td>
<td>208 $^{ab}$</td>
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<tr>
<td>17 weeks</td>
<td>9309 $^a$</td>
<td>6 $^a$</td>
<td>8 $^a$</td>
<td>100.0 $^a$</td>
<td>65 $^a$</td>
<td>214 $^a$</td>
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<tr>
<td>'Fall Gold'</td>
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<td>80.0 $^a$</td>
<td>28 $^{ab}$</td>
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<td>8 $^b$</td>
<td>82.0 $^a$</td>
<td>22 $^b$</td>
<td>126 $^b$</td>
</tr>
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<td>17 weeks</td>
<td>2000 $^a$</td>
<td>10 $^a$</td>
<td>8 $^b$</td>
<td>96.0 $^a$</td>
<td>36 $^a$</td>
<td>150 $^a$</td>
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<td>8 $^a$</td>
<td>97.0 $^a$</td>
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<td>131 $^a$</td>
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<td>8 $^a$</td>
<td>99.3 $^a$</td>
<td>58 $^a$</td>
<td>146 $^a$</td>
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<tr>
<td>17 weeks</td>
<td>3392 $^a$</td>
<td>7 $^a$</td>
<td>7 $^a$</td>
<td>95.2 $^a$</td>
<td>53 $^a$</td>
<td>147 $^a$</td>
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</table>

$^\dagger$: Leaf area of the leaves developed after the temperature stress period

$^\text{Y}$: Data were log transferred prior to statistical analysis but original mean values are shown

Different letters within the same column and cultivar indicate significant difference at $P < 0.05$ by Tukey-Kramer test
cultivars also chlorophyll content. A decline in the efficacy of photosystem II under elevated temperature regimes at midday and partial recovery at evening in ‘Polka’ may indicate coordinated changes in the photosynthetic apparatus and processing that might help plants to survive in heat stress. An extended cold-storage period suppresses lateral shoot formation and promotes the number of flower buds per lateral in ‘Autumn Bliss’ and ‘Fall Gold’. Moreover, heat stress enhances early flowering in ‘Autumn Bliss’ and delays it in ‘Autumn Treasure’, indicating distinct cultivar differences. In commercial production, this information may be useful for manipulating and optimizing fruit production in glasshouses and outside in warmer regions. Therefore evaluation of raspberry germplasm for cultivation in warmer areas should be performed. However, we suggest that longer stress exposure than the seven day period and above 37 °C should also be examined to understand the effects of heat stress in detail.

Acknowledgements

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References


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Chapter 10: Paper II

Effect of short-term exposure to high temperature on gene expression in four raspberry 
(Rubus Ideaus L.) cultivars

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(Submitted to the Journal of Horticulture Science and Biotechnology)
Effect of short-term exposure to high-temperature on gene expression in leaves of four raspberry (Rubus idaeus L.) cultivars

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Running head: Temperature stress and gene expression in raspberry

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SUMMARY

The effect of a high-temperature stress (27°C or 37°C for 24 h) on gene expression profiles in the annual-fruiting raspberry (*Rubus idaeus* L.) cultivars ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’ and ‘Polka’ were evaluated at the flower initiation stage were evaluated using a customised *Rubus* microarray. Significantly affected genes were obtained by pairwise t-tests using ‘volcano plots’ for each cultivar x treatment. A 10°C elevation in temperature altered the levels of expression of 40 genes (38 were down-regulated and two were up-regulated) among the four cultivars. ‘Volcano’ filtering identified 12 common candidate genes that were modulated differentially in ‘Autumn Bliss’ and ‘Erika’ at 37°C compared to 27°C. Two aquaporin genes (*PIP1* and *TIP2*) were down-regulated in ‘Autumn Bliss’, but up-regulated in ‘Autumn Treasure’, ‘Polka’ and ‘Erika’. The down-regulated genes included those encoding major latex-like protein (MLPs), plasma membrane proteins (PMPs), cysteine rich proteins, and other stress-related proteins. Validation by real-time quantitative RT-PCR indicated subtle changes in gene expression differences suggesting a mild response to heat stress. This study used molecular tools to increase our understanding of, and to identify candidate genes involved in, the heat stress response of four annual-fruiting raspberry cultivars.
Most raspberry (*Rubus idaeus* L) production is concentrated in cold temperate areas of the World. However, annual-fruiting raspberries tend to be grown in warmer regions such as Southern Europe where the summer temperatures are relatively high (Graham *et al*., 2007; Graham and Jennings, 2009). Outdoor temperatures have been predicted to fluctuate more and may increase further due to future climate change. Elevated ambient temperatures have adverse effects on plant physiology, biochemistry and metabolism (Wahid *et al*., 2007), modulate gene expression, concentration and properties of proteins and metabolites (Ahuja *et al*., 2010), and cause protein aggregation and denaturation (Berry and Bjorkman, 1980). The optimum temperature for annual-fruiting raspberry cultivation ranges from 16° - 24°C, but some cultivars such as ‘Polka’, grow successfully even at 30°C (Sonsteby and Heide, 2009). Most raspberry cultivars are poorly adapted to the high temperatures that may occur during the Summer months and in protected cultivation like tunnels and greenhouses (Ballington and Fernandez, 2008). Gotame *et al*. (2013) indicated that temperatures above 32°C generally reduced the maximum photosynthetic efficiency in annual-fruiting raspberry leaves. Total protein concentration decreased and peroxidase activities increased when strawberry leaves were exposed to temperatures > 25°C (Gulen and Eris, 2004). As with other abiotic stresses, growing plants at high temperatures also triggers adjusting or defence mechanisms by changing the levels of some gene transcripts, and creating signals for metabolic adjustment (Mittler *et al*., 2012). Heat stress also causes alterations in the expression of genes for osmoprotectants, detoxifying enzymes, solute transporters, and regulatory proteins. For example, studies on grapevine leaves reported that heat stress caused a two-fold up-regulation of those genes for important traits which have putative involvement in cell rescue (i.e., antioxidant enzymes), protein fate (i.e., heat shock proteins), primary and secondary metabolism, transcription factors and signal transduction compared to recovery-regulated genes (Liu *et al*., 2012).

Microarrays are powerful tools to measure changes in the expression of large number of genes of interest in different tissues and at different stages of development simultaneously (Slonim and Yanai, 2009). Gene expression profiling using microarrays has increased our understanding of several important biological processes in crops such as berries (e.g., strawberry, blackcurrant and raspberries) under specific physiological conditions (Aharoni and O’Connell, 2002; Mazzitelli *et al*., 2007; Chen *et al*., 2010; Hedley *et al*., 2010). Moreover, microarrays have also been used to measure changes in gene expression patterns
(the transcriptome) in horticultural crops affected by high temperatures, for examples, Chinese cabbage (Yang et al., 2006), tomato (Frank et al., 2009), sunflower (Hewezi et al., 2008), potato (Ginzberg et al., 2009), and grapevine (Liu et al., 2012). Microarrays were also used to identify candidate genes associated with the release of bud dormancy in raspberry (Mazzitelli et al., 2007) and in blackcurrant (Hedley et al., 2010). These studies related the patterns of expression of specific genes to physiological changes under normal growing conditions.

Changes in gene expression in response to high temperatures in raspberry leaf tissues, particularly at the floral initiation stage, have not yet been reported. In this study, we used a Rubus microarray to study the effect of high temperatures (27°C or 37°C) for only a short duration (24 h) on the total gene expression levels in four annual-fruiting raspberry cultivars (‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, and ‘Polka’). In the previous study, ‘Autumn Bliss’ was the most susceptible and ‘Autumn Treasure’ and ‘Erika’ showed some degree of tolerance, based on chlorophyll fluorescence measurement and chlorophyll pigment analysis (Gotame et al., 2013). Therefore, in this study, we selected and compared the most heat susceptible and heat tolerant cultivars to identify changes in gene expression in raspberry leaves under high temperature stress.

MATERIALS AND METHODS

Plant material and treatment

Four annual-fruiting raspberry cultivars, (‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, and ‘Polka’) were propagated in 3.5 l pots, in duplicate, and grown under greenhouse conditions at 20° +5°C with a 14 h photoperiod until flower initiation. Fertigation was done once a day to achieve pot capacity using a fertiliser solution with an electrical conductivity (EC) of 2.16 mS cm⁻¹ containing 40 mg l⁻¹ NH₄-N, 165 mg l⁻¹ NO₃-N, 44 mg l⁻¹ phosphorus (P), and 257 mg l⁻¹ potassium (K). When the plants reached the stage of floral initiation (usually 7 weeks after root sprouting), they were transferred to climate chambers (MB-Teknik, Broendby, Denmark) set at 27°C and 37°C with a 14 h photoperiod at a constant 350 µmol m⁻² s⁻¹ photosynthetically active radiation. The relative humidity of 60 ± 5%. All plants were watered with a complete nutrient solution to pot capacity at 08:00 h and 17:00 h each day. The youngest leaf at the top of one shoot on each plant was collected after 24 h exposure to 27°C or 37°C and immediately frozen in liquid nitrogen, and stored in -80°C before being
freeze-dried (Christ Gamma 1-20 LSC, SciQuip Ltd, Shropshire, SY4 5NU, UK) for total RNA extraction.

**Total RNA isolation**

Total RNA was extracted from each freeze-dried leaf (0.1 g) sample collected immediately after 24 h heat exposure using the RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's recommendations with the addition of 45 µl (10%) (v/v) RNA Isolation Aid (Ambion Life Technologies Ltd, Paisley PA4 9RF, UK) and 4.5 µl (1%) (v/v) β-mercaptoethanol (Sigma-Aldrich Co. Ltd., Gillingham Dorset SP8 4XT, UK) to 450 µl RNeasy Lysis Buffer (RLT). The concentration and purity of each total RNA samples were analysed spectrophotometrically at 230 nm, 260 nm, and 280 nm using a NanoDrop ND-1000 Full-spectrum UV-Visible Spectrophotometer (ThermoFischer Scientific, Epsom, UK). An A$_{260}$: A$_{280}$ ratio of 1.8 - 2.0 indicated RNA of adequate quality. The integrity of each RNA was also checked by 2% (w/v) agarose gel electrophoresis and using an Agilent Bio-analyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) which provides an objective RNA Integrity Number (RIN) from 1 (degraded) to 10 (intact). RNAs extracted from leaf material of all four raspberry cultivars had A$_{260}$: A$_{280}$ ratio in the range of 1.8 - 2.0 and their RIN values were usually > 7.0, indicating high quality. The total RNA samples (100 mg) were stored at -80°C in batches.

**Microarray processing and data analysis**

A simple, pairwise microarray experimental design was devised to use the *Rubus* Agilent 60 K microarray developed at the James Hutton Institute, (Invergowrie, UK) designed from a unigene set derived from RNAseq datasets. This custom microarray was designed from existing sequence resources, comprising transcript sequences isolated from a range of *Rubus* tissues, developmental stages and conditions, including developing fruit and buds. This set was composed of sequences originating from four sources: (i) Roche 454 transcripts (52,263); (ii) Illumina GAII transcripts (118,275); (iii) Sanger Expressed Sequence Tags (4,360) and; (iv) BAC coding sequences (1,425). In total, 176,833 sequences were assembled using CAP3 software, generating 41,155 contigs and 22,098 singletons. These sequences were BLASTx searched against known plant polypeptide sequences to identify the top protein homologues which, along with the presence of a polyA or polyT tract, enabled determination of predicted orientation for 55,920 unigenes. Using eArray online software (https://earray.chem.agilent.com/earray/) with default parameters, a total of 55,708
oligonucleotide probes (one 60mer per unigene) were designed for generation of a custom Agilent microarray in 8x 60k format (JHI_Ri_60k_v1; Agilent array design AMADID 035443; ArrayExpress https://www.ebi.ac.uk/arrayexpress accession # A-MEXP-2373. In total, 16 RNA samples were processed (four cultivars x two temperatures (27°C and 37°C) x two biological replicates) in a Two-Colour Microarray design (27°C verses 37°C for each sample, including dye-swaps where each biological replicate was labelled with Cyanine3 (rep 1) or Cyanine5 (rep 2) for each sample. Total RNA (100 ng per sample) was labelled using the standard recommended procedures (Agilent Low-Input QuickAmp RNA Labelling Kit). Labelling efficiencies were checked following purification on a NanoDrop ND-1000. Hybridisation of each RNA sample to the Rubus microarray was performed overnight, as recommended, using the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent Version 6.5). The microarrays were scanned at two wavelengths for data acquisition (for Cyanine3 and Cyanine5 dye) using an Agilent Microarray Scanner (G2505B), resulting in single tiff images for each array.

The raw data were extracted from each microarray using Agilent Feature Extraction software (version 10.7.3.1). Data were imported into Agilent GeneSpring software (version 7.3.1) for subsequent quality control (QC) filtering and analysis. Data were normalised using the LOWESS algorithm (Locally Weighted Polynomial Regression) to balance for dye and signal intensity within and between microarrays. The data were re-imported into GeneSpring as single-colour data to permit for more flexible analysis. Consistently low-expressed genes were filtered out from the total dataset (55,708 probes), leaving 39,049 probes with signals (> 50) in at least one replicate. Comparisons were then made between the two temperature treatments (27°C or 37°C) for each cultivar using ‘volcano plots’ with threshold a two-fold change and Student’s t-test (two-fold difference in expression level with a \( P \leq 0.05 \); default parameters in Genespring). Probes from the ‘volcano filtering’ were clustered using Gene Tree and Pearson’s correlation coefficient (default parameters in Genespring) to generate a heat map. Unique and overlapping genes between the lists of up-regulation and down-regulation were selected using a Venn Diagram (data not shown). Sequences related to the microarray probes were obtained using a BLASTN search of non-redundant nucleotide (nr/nt) databases against the National Centre for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Real-time quantitative RT-PCR
Reverse transcription of a standard amount (1.0 µg) of total RNA per sample of ‘Autumn Bliss’ and ‘Erika’ was performed using the QuantiTect Reverse Transcription kit (Qiagen) with oligo d(T) and random hexamers, as primers according to the manufacturer’s instructions. The cDNA thus synthesised was diluted to 10 ng µl$^{-1}$ in 100 µl with sterile distilled water. Primers were designed for four candidate genes (for a plasma membrane protein, an aquaporin, a cysteine protein, and a major latex like protein; Table I, Supplementary file S1) were selected for further study on the basis of showing a significant differences in levels of expression between the four cultivars in the microarray experiment.

Several reference genes for transcript normalisation in Rubus were also selected from the literature (Czechowski et al., 2005) and the stability of the expression profiles of the equivalent genes was examined from the array. The gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an appropriate internal and later a qRT-PCR control gene. Several sets of primers and probes were designed for conventional PCR and for real-time qRT-PCR using Primer3 (Rozen and Skaletsky, 2000) or the UPL (Universal Probe Library) Assay Design Centre, and recommended parameters from Roche Diagnostics Ltd. (www.roche-applied-science.com/shop/CategoryDisplay?catalogId=10001&tab=&identifier=Universal+Probe+Library&langId=-1).

Conventional PCR reactions were performed initially in order to confirm that each primer pair amplified a single product of the correct size and one µl of cDNA (10 ng µl$^{-1}$) was added to each 24 µl master mix consisting of 1 x Go Taq Reaction Buffer (Promega, Southampton, UK), 0.2 mM each dNTP (Promega), 0.3 µM each primer (Eurogentec Ltd., UK), and 1.0 Unit Go Taq DNA Polymerase (Promega). Each PCR amplification was based on an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a final elongation step at 72°C for 3 min using a Veriti 96-Well Thermal Cycler (Applied Biosystems Ltd., Warrington, UK). The PCR products (in 10 µl) were analysed in 2.0% (w/v) agarose gels containing 0.5 µg ml$^{-1}$ ethidium bromide in 0.5x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) buffer.

In an effort to produce consistent, high quality (‘gold standard’) data from the qRT-PCR studies, the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009) were followed. All qRT-PCR reactions were performed with 2 µl cDNA (at 10 ng µl$^{-1}$) added to 23 µl of FastStart TaqMan Probe Master mix with Rox reference dye (Invitrogen, Life Technologies Co. UK), and run on an
automated ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Inchinnan Business Park, Paisley PA4 9RF, UK) using a standard mode and a three-step cycle: 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 60°C for 1 min. All the PCR primers were added to an optimal final concentration of 900 nM, and the UPL probes were used at 100 nM. In those cases where UPL probes could not be used, the same volumes and concentrations and cycle conditions were applied using the Power SYBR Green PCR Master Mix (Applied Biosystems) with a ‘melt curve analysis’ to detect any non-specific amplification. All PCR reactions were repeated in a three technical and two biological replications with independent cDNA samples.

Normalized gene expression levels of the aquaporin (TIP2) and the plasma membrane protein in ‘Autumn Bliss’ and ‘Erika’ grown at 27°C and 37°C from real-time qRT-PCR were according to Pfaffl (2001) comparing the target gene to the reference gene (GAPDH). This determined the relative quantity of the candidate genes in comparison to the GAPDH reference gene for normalization of the data. This was necessary because the relative efficiencies of all qRT-PCR assays were not equal, and the model incorporated the reaction efficiencies of both the candidate gene and the reference gene. The qRT-PCR efficiencies were calculated from the slope of each standard curve (Ct values versus log [cDNA]; Vaerman et al., 2004) by testing a pooled cDNA mixture (10 ng µl⁻¹) consisting of ‘Autumn Bliss’ and ‘Erika’ cDNAs (exposed to 27°C and 37°C) over a five-fold dilution series (20, 4, 0.8, 0.16, 0.032, and 0.0064 ng l⁻¹) under the PCR conditions described above. The corresponding PCR efficiency (E) was calculated according to the equation

\[
E = 10^{[-1/slop]} -1;
\]

Where efficiencies between 80 - 110% were considered acceptable.

All PCR assays produced a standard curve within the dilution range (20.0 - 0.0064 ng l⁻¹) with high linearity (Pearson’s correlation coefficient (r) values > 0.99). The amplification of a single PCR product was also verified in a random selection of samples after each real-time qRT-PCR separated by agarose gel electrophoresis. All relative levels of gene expression were compared to those for ‘Autumn Bliss’ at 27°C, and statistical analysis using General Analysis of Variance was performed using GeneStat 15.1 (GenStat V10 VSNi, Hemel Hempstead, UK).

*Genomic DNA extraction, PCR, and sequencing*
Genomic DNA for sequencing was extracted from fresh leaf material (1.0 g) from eight raspberry cultivars (‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, ‘Glen Ample’, ‘Glen Fyne’, ‘Octavia’, ‘Polka’ and ‘Tulameen’) using the optimised manual protocol for raspberry. The DNA extraction buffer was prepared by dissolving 2.0 g CTAB, 1.2 g Tris-HCl (100 mM, pH 8.0), 8.2 g NaCl (1.4 M) and 0.74 g EDTA (20 mM, pH 8.0) in 100 ml water and autoclaving it at 121°C under 2 atmospheric pressure for 45 min. Dithiothreitol (DTT; Sigma-Aldrich Co. Ltd.) was immediately then added to the extraction buffer to 0.1% before DNA extractions. Fresh leaf tissue from each cultivar was ground with a mortar and pestle in liquid nitrogen and transferred to a 15 ml sterile tube. A spatula tip of polyvinylpyrrolidone (PVP; Sigma-Aldrich Co. Ltd.) was added followed by 5 ml of DNA extraction buffer (above) were added and the contents were vortex mixed before incubation at 65°C for 30 min. A mixture of 24:1(v/v) chloroform: IAA (Sigma-Aldrich Co. Ltd.) was added (each 7.5 ml per extraction) and sample was shaken for 15 min at room temperature followed by centrifugation for 15 min at 2,300 x g (4°C). The aqueous layer was filtered through one layer of muslin cloth and an equal volume of ice-cold isopropanol was added before incubation for 15 min at room temperature followed by 30 min at 0°C. The DNA was pelleted by centrifuging for 15 min at 2,300 x g (4°C) and re-suspended in 0.75 ml sterile water.

Various PCR primer combinations (Table I) for the candidate genes [i.e., plasma membrane protein and aquaporin (TIP2)] were first tested on the DNA samples extracted from the all eight raspberry cultivars in order to generate products for sequencing to identify sequence polymorphisms and associated markers for heat stress. Conventional PCR was performed with 100 - 150 ng µL⁻¹ gDNA as described previously, and the products were separated by electrophoresis in a 2% (w/v) agarose gels. Single PCR product was treated with ExoSAP-IT (USB Products, Affymetrix, Inc., Ohio, USA) according to the manufacturer’s instructions and sequenced in both directions by Sanger sequencing. The primer pairs used to generate products for sequencing were **PMPF1** plus **PMPR1** (product = 475 bp) and **Aqua1F1** plus **Aqua1R2** (product = 663 bp) for the plasma membrane protein and aquaporin genes, respectively (Table I).
Table I
Oligonucleotide primers and probes used in this study

<table>
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<th>Candidate gene (Accession number)</th>
<th>Primer Sequences (5'→3')</th>
<th>Amplicon size (bp)</th>
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<td>pmpOR</td>
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</tr>
<tr>
<td>cyspUPLF</td>
<td>Fwd: inner</td>
<td>CACACTTTCTTGGCCTCTC</td>
</tr>
</tbody>
</table>
For consistency, the primer pairs for real-time qRT-PCR were designed (if possible) to cover the 60-mer probe sequence used in the microarray experiment.

**GAPDH**, glyceraldehyde-3-phosphate dehydrogenase gene.

UPL, Universal Probe Library.

<table>
<thead>
<tr>
<th>Gene/Reference</th>
<th>Primer Pair Information</th>
<th>Probe Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major latex like protein (JHI_Ri_ASM02Jun2011_MMB_34674)</td>
<td>mlpOF (Fwd: outer)</td>
<td>GAATACTTCACGCGCAGACC</td>
</tr>
<tr>
<td></td>
<td>mlpOR (Rev: outer)</td>
<td>CAAGGGATGGAGACCAGATG</td>
</tr>
<tr>
<td></td>
<td>mlpUPLF (Fwd: inner)</td>
<td>GGAAGCTAAAGTTGCCAAGG</td>
</tr>
<tr>
<td></td>
<td>mlpUPLR (Rev: inner)</td>
<td>GAGGAAATGGAGGAGGCAAT</td>
</tr>
<tr>
<td>UPL 135</td>
<td>gapdhOF (Fwd: outer)</td>
<td>TGAAGATGGAAGGCTTTGCT</td>
</tr>
<tr>
<td>Reference gene</td>
<td>gapdhOR (Rev: outer)</td>
<td>AACCCCATCAAATTTTGTTTT</td>
</tr>
<tr>
<td>(GAPDH)</td>
<td>gapdhUPLF (Fwd: inner)</td>
<td>TGGTCTTCTCTCGAGTTG</td>
</tr>
<tr>
<td></td>
<td>gapdhUPLR (Rev: inner)</td>
<td>GCGGAACTCGAAAACTAAAAGG</td>
</tr>
<tr>
<td>UPL 14</td>
<td></td>
<td>TCTCCCAAG</td>
</tr>
</tbody>
</table>
Sequences were analysed and edited manually using Sequencher 4.9 (DNA Codes Corp., Ann Arbor, MI, USA) and aligned using the ClustalW2 multiple sequence alignment programme (www.ebi.ac.uk/Tools/msa/clustalw2/) to identify any sequence polymorphisms.

**Linkage map construction for the aquaporin candidate genes PIP1 and TIP2**

An up-dated marker map was produced to include both aquaporin genes (PIP1 and TIP2) using Join Map 3.0 (Van Ooijen and Voorrips, 2001) after PCR amplification and scoring all 188 individuals in the 'Latham' / 'Glen Moy' mapping population. Details of the map construction are given in Graham et al. (2009).

**RESULTS AND DISCUSSION**

**Expression of genes responsive to heat stress**

Variations in gene expression in the leaves of four annual-fruiting raspberry cultivars in response to 24 h of high temperatures were measured using the Agilent *Rubus* 60 K microarray with 55,708 probes. Initial quality control steps left 39,049 probes with signal (> 50) in at least one replicate for each sample. Statistical analyses (Student’s *t*-test) using ‘volcano plots’ to combine strict selection criteria (two-fold change in expression level with a *P* value ≤ 0.05) was carried out between temperature treatments (27°C and 37°C) for all four cultivars, ‘Autumn Bliss’, ‘Autumn Treasure’, Erika’, and ‘Polka’.

Pairwise *t*-tests using ‘volcano plots’ identified 427 differentially expressed genes for ‘Autumn Bliss’ and 229 genes for ‘Erika’, and revealed 12 candidate genes were common and down-regulated at 37°C compared to 27°C (data not shown).

Microarray probes from the ‘volcano’ filtering step were clustered using a Gene Tree and Pearson’s correlation coefficient to generate a heat map to aid in the selection of differently expressed candidate genes among the cultivars following heat treatment (Figure 1).
FIG. 1

Heat map of volcano filtered probes that changes significantly between the two temperature treatments (27°C or 37°C) in four annual-fruiting raspberry cultivars ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, and ‘Polka’. Red, up-regulated; Green, down-regulated; Black, no changed. The selected probe pairs highlighted on the right indicates of 40 genes significantly affected by high temperatures and subsequently studied.
From the heat map, a total of 644 genes were differentially expressed between temperatures in at least one cultivar. ‘Erika’ and ‘Autumn Treasure’ showed elevated expression of 38 genes compared to ‘Autumn Bliss’ and ‘Polka’ (Figure 2). Potential candidate genes were selected on the basis of specific gene expression patterns in the Gene Tree in a region of significantly changing profiles (Figure 1) and using the ‘volcano’ filtering criteria. A total of 40 genes were significantly affected by high temperature exposure for 24 h. Graphical views of expression profiles of all genes for each cultivar are represented in Figures 2 and 3. Among these probe sets that showed differential expression during heat treatment, 38 were down-regulated in all cultivars and two aquaporin genes (\textit{PIP1} and \textit{TIP2}) were up-regulated in ‘Polka’, ‘Autumn Treasure’ and ‘Erika’ but not in ‘Autumn Bliss’ and there were significant differences in gene expression levels between treatments (Supplementary file S1).

![Graphical view of the 38 significantly up-regulated and two down-regulated genes in the four raspberry cultivars ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, and ‘Polka’ following 24 h growth at 27°C or 37°C.](image)

**FIG. 2**

Graphical view of the 38 significantly up-regulated and two down-regulated genes in the four raspberry cultivars ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Erika’, and ‘Polka’ following 24 h growth at 27°C or 37°C.

A BLASTN search of sequences closely related to the microarray probes indicated that the list of potential candidate genes belonged to different functional categories and
included several examples each of major latex-like protein (MLP), plasma membrane protein (PMP), aquaporins, and other stress related protein (Supplementary file S1). The list of down-regulated genes contained many of the genes known to encode for metal binding (metallothionein-like protein), ubiquinone reduction (electron transfer flavoprotein-ubiquinone oxido-reductase), flower development (latex-like protein), solute transport (PMP) and Rubisco turn over in leaves (cysteine protein). Moreover, based on current knowledge, the 12 genes that were down-regulated in both ‘Autumn Bliss’ and ‘Erika’ (as identified by microarray annotation) encode the same function, for example, major latex-like protein (MLP). Moreover, a BLASTN search also showed five of the genes responsive to the heat stress did not match any genes of known functions.

![Graphical view of pattern of expression of the two aquaporin genes (PIP1 and TIP2) in the four raspberry cultivars ‘Autumn Bliss’, Polka’, ‘Autumn Treasure’ and Erika’ after 24 h growth at 27°C or 37°C.](image)

**FIG. 3**

Graphical view of pattern of expression of the two aquaporin genes (PIP1 and TIP2) in the four raspberry cultivars ‘Autumn Bliss’, Polka’, ‘Autumn Treasure’ and Erika’ after 24 h growth at 27°C or 37°C.

*Real-time quantitative RT-PCR validation*

In order to validate results obtained from the microarray experiment, specific qRT-PCR assays were designed for selected candidate genes representing different biological functions (Table I) and showing a relatively higher-fold change in expression. The
chlorophyll concentration measurement showed that ‘Autumn Treasure’ and ‘Erika’ were similar but different with ‘Autumn Bliss’ and ‘Polka’ when grown at 27°C and 37°C (Gotame et al., 2013). Therefore, ‘Autumn Treasure’ and ‘Polka’ were removed from further gene expression analysis using qRT-PCR. Moreover, ‘Erika’ and ‘Autumn Bliss’ were selected as they are genetically related; ‘Erika’ being a selection from open-pollinated ‘Autumn Bliss’ (Nikki Jennings, personal communication). These assays were first tested in the more genetically related but contrasting cultivars, ‘Autumn Bliss’ and ‘Erika’. From the 40 microarray probe sets, the four candidate genes selected were plasma membrane protein, aquaporin (TIP2), cysteine protein and major latex like protein (Table I).

UPL Design Centre software (Roche) was used to design UPL probe-based assays for real time qRT-PCR and ensure common thermal cycling parameters. Although all primer pairs successfully amplified a single product for all four candidate genes during conventional PCR (Supplementary Figure 1), the assays designed for the plasma membrane protein, cysteine protein and major latex-like protein gene sequences all failed to produce a signal in the real-time qRT-PCR format. Further investigation using outer primer pairs (Table I) and sequencing of products for each assay and cultivar revealed sequence variation in the region of the UPL probe design that explain the absence of a signal (data not shown). Moreover, the microarray probes were designed to a single Rubus cultivar ‘Glen Moy’ and since distantly related genotypes were used in this study, this explains the absence of an amplification signal. This was not the case for the aquaporin or the GAPDH reference gene assays which worked successfully with the UPL probe in the real-time qRT-PCR format. Additional optimisation with the SYBR Green master mix (see materials and methods) was subsequently tested in order to use the same primer sequences and this was successful for the plasma membrane protein gene, but not for the cysteine and major latex-like protein genes; the latter two assays failed to operate efficiently ($E < 65\%$) and were removed from the validation procedure.

This highlighted the need to be aware that the occurrence of small sequence variation amongst different raspberry genotypes can result in failure to detect signals in the real-time qRT-PCR format. The occurrence of single nucleotide polymorphisms (SNPs) or indels in annealing regions of primers or probes may hamper efficient annealing of the primer/probe or prevent amplification of a variant allele.

The normalized expression levels of the aquaporin (TIP2) increased by 1.3-fold in ‘Autumn Bliss’ and three-fold in ‘Erika’ grown at 37°C compared to 27°C. However, in contrast, the expression levels of the plasma membrane protein gene decreased by two-fold in ‘Autumn Bliss’ and three-fold in ‘Erika’ grown at 37°C compared to 27°C (Figure 4).
Analysis of variance from the qRT-PCR results revealed that ‘Autumn Bliss’ and ‘Erika’ were significantly different in their expression ($P \leq 0.05$).

The two candidate genes, aquaporin (TIP2) and plasma membrane protein, were first studied due to a differential expression in ‘Autumn Bliss’ and ‘Erika’ as detected from the microarray analysis. Differential gene expression was subsequently compared and validated with the alternative technique of real time qRT-PCR, and there was agreement by both procedures apart from the expression signals of aquaporin (TIP2) in ‘Autumn Bliss’ at 27°C and 37°C (Figure 4). Although the magnitude of fold-change differed in both ‘Autumn Bliss’ and ‘Erika’, the direction of fold-change (down-regulated) was similar in $PMPs$ gene.

**FIG. 4**

Comparisons between levels of gene expression derived by qRT-PCR and microarray analysis (raw values were used from Supplementary file 1). Bars shows the fold-change in gene expression of the aquaporin gene (TIP2), panel A), and the plasma membrane protein
gene (*PMP*), panel B) in response to growth at 27°C or 37°C for 24 h in ‘Autumn Bliss’ and ‘Erika’ raspberry.

**Candidate gene mapping and sequencing to identify heat stress markers**

An updated linkage group map was produced to include both the aquaporin genes (*PIP1* and *TIP2*) after scoring in the 188 individuals of the 'Latham' / 'Glen Moy' mapping population (Figure 5).

![Figure 5: Genetic linkage map positions of the raspberry aquaporin (*PIP1*) and (*TIP2*) candidate genes located on Linkage Group 3.](image)

Genetic linkage map positions of the raspberry aquaporin (*PIP1*) and (*TIP2*) candidate genes located on Linkage Group 3.

Both aquaporin genes mapped at almost identical positions on Linkage Group 3 of the seven *Rubus idaeus* groups, indicating that these related genes in terms of sequence homology (60%) may also be members of the same gene family. Linkage Group 3 contained genes associated with fruit quality traits such as flavour, colour, softening, and ripening (Graham *et al.*, 2009), and we can speculate that the two aquaporin genes may play additional roles in fruit quality as well as for a heat-stress response.
Sequences generated from the PCR products of the two heat stress candidate genes [i.e., the plasma membrane protein and aquaporin (*TIP2*)] were obtained from all eight raspberry cultivars to identify polymorphisms and as potential markers for heat stress.

**Discussions**

The expression of the shared set of genes in ‘Autumn Bliss’ and ‘Erika’ may indicate that these two cultivars can have repression of the same set of genes, probably via common and similar signalling pathways regulating the set of 12 genes.

Down-regulation of electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) was observed in all four raspberry cultivars in microarray experiment. The ETF-QO links the oxidation of fatty acids and some amino acids to oxidative phosphorylation in the mitochondria (Ishizaki *et al.*, 2005). It has been also reported that ETF-QO catalyses the transfer of electrons from ETF to ubiquinone, reducing it to ubiquinol. Oxidative phosphorylation involves the reduction of O$_2$ to H$_2$O with electrons donated by NADH (i.e. transfers electrons from NADH to oxygen) and FADH$_2$ which releases free energy and synthesises ATPs that finally completes the oxidation of sucrose (Taiz and Zeiger, 2006).

There was also a down-regulation of metallothionein-like genes. Metallothionein-like genes are metal binding, cysteine rich metal binding proteins involved in metal ion detoxification (Cobbert and Goldsborough, 2002) and have also been implicated in the regulation of ATP production to reduce metal-induced oxidative stress (Thomas *et al.*, 2005).

Down-regulation of a zinc finger (C$_3$HC$_4$-type ring finger) family protein gene was also observed at 37°C and also reported by Liu *et al.* (2012) in grapevine with a B-box type zinc finger-containing protein and was also down-regulated following high temperature treatment. This gene down-regulation seems to be a common adaptive response that enables plants to cope with new environmental conditions, possibly in order to conserve energy and used to activate heat tolerance responses.

Interestingly, two aquaporin genes (*PIP1* and *TIP2*) from the ‘volcano’ list were down-regulated in ‘Autumn Bliss’ but up-regulated in ‘Autumn Treasure’, ‘Polka’ and ‘Erika’. These genes were validated with the alternative technique of real time qRT-PCR, and there was agreement by both procedures apart from the expression signals of aquaporin (*TIP2*) in ‘Autumn Bliss’ at 27°C and 37°C. This slight discrepancy may be explained by the different normalization procedures and or the measurement of different but related aquaporin genes in ‘Autumn Bliss’ by both methods. The plasma membrane aquaporin and aquaporin
homologs are termed PIPs (plasma membrane intrinsic proteins), whereas tonoplast aquaporin and aquaporin homologs are named TIPs (tonoplast intrinsic proteins) (Johanson et al., 2000; Johansson et al., 2001). It was reported that five aquaporin subfamilies were identified in plants based on DNA similarities. The aquaporin facilitates the efficient transport of water molecules across membranes, play a role in controlling intercellular water movement and facilitate passive exchange of water, compatible solute distribution and gas transfer across membranes (Johanson et al., 2001). A few PIPs have also been reported to be involved in CO₂ permeability of cells. The plasma membrane determines the internal mesophyll CO₂ conductance (gₘ) due to the mesophyll cells imposing resistance to CO₂ diffusion (as reviewed by Katsuhara et al., 2008). Aharon et al. (2003) also observed an increase in photosynthetic rate of transgenic tobacco plants up-regulating Arabidopsis PIP1.

Expression of aquaporin isoforms are known to be affected by environmental stresses (as reviewed by Jang et al., 2007). The stress may either increase the transcription of the aquaporin gene or it may increase the activity of existing aquaporin. Studies have reported the expression of aquaporin genes under a wide range of biotic and abiotic stresses including heat and drought stresses (Hewezi et al., 2008). For example, PIPs are involved in the regulation of gₘ as a rapid response in drought conditions (Flexas et al., 2002). Jang et al. (2007) reported that overexpression of PIP1;4 or PIP2;5 isoforms suppressed plant growth under drought stress but no effect was reported under normal conditions. Aharon et al. (2003) also showed a negative role of an aquaporin during drought stress. Under heat stress at day time, there is an increase in transpiration which induces water deficit in plants and causes a decrease in leaf water potential (Tsukaguchi et al., 2003). Changes in aquaporin expression may also be related to the need to control water movement between storage tissues and rapidly growing and expanding tissues. Mazzitelli et al. (2007) observed a down-regulation of expressed sequence tags with similarity to an aquaporin gene in raspberry buds during the transition from endo- to para-dormancy. An influence of aquaporin expression on photosynthetic performance of plants was also reported (Aharon et al., 2003; Flexas et al., 2006). Aharon et al. (2003) performed chlorophyll measurement in tobacco plants over-expressing an Arabidopsis PIP1b gene and reported a positive correlation with expression of PIP1b and maximum quantum efficiency of dark adapted leaves. Flexas et al. (2006) also reported that Nicotiana tabacum L. aquaporin (NtAQP1) contributes to CO₂ conductivity of mesophyll cells (gₘ) in tobacco. The effects of drought and heat stress on cereals are also interlinked and suggesting a common mechanism for heat, drought and other osmotic stress...
Up-regulation or down-regulation of aquaporin was reported to affect the leaf cell water permeability, water loss rate, stomatal conductance and overall leaf function (Heinen et al., 2009). The TIPs are a major component of the tonoplast and provide a quick equivalence of osmotic balance between cytosol and vacuolar lumen to prevent plasmolysis under hypertonic conditions (Katsuhara et al., 2008). Down-regulation of TIPs may be related to storage of water in vacuoles in drought tolerant cultivars or to low water stress conditions and vice versa. In our observation, the differences in heat tolerance in ‘Autumn Bliss’ and ‘Erika’ may be associated with multiple processes and mechanisms involving heat response proteins, transcription factors and stress related genes.

The down-regulation of 12 isoforms of MLPs were observed all four raspberry cultivars which encoded the similar function, and studies reported that MLPs are specifically related to with fruit and flower development and in a pathogen defence response (Lytle et al., 2009).

Linkage Group 3 contained genes associated with fruit quality traits such as flavour, colour, softening, and ripening (Graham et al., 2009), and we can speculate that the two aquaporin genes may play additional roles in fruit quality as well as for a heat-stress response.

However, although sequence polymorphisms (indels or single nucleotide polymorphisms (SNPs)) were observed in the portion of both gene sequences examined after alignment (data not shown), no clear association with either the heat-tolerant or heat-susceptible for the eight raspberry cultivars were found. Additional germplasm and portions of both candidate gene sequences and a larger segregating population or genotypes for association mapping will be needed to examine and identify potential polymorphic markers for heat-stress.

We have reported the first use of the Rubus microarray for high-throughput gene expression analyses to determine the relative abundance of mRNAs expressed in four heat-stressed raspberry cultivars. The microarrays revealed that a major response of raspberry genes to high temperatures for a 24 h short period involved down-regulation of defence-related genes and up-regulation of two aquaporin related genes.

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Chapter 11: Manuscript III

Influence of production methods on yield and quality parameters of commercial raspberry cultivars grown under high tunnels in Danish conditions

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(Prepared for submission to Scientia Horticulturae)
Influence of production methods on yield and quality parameters of commercial raspberry cultivars grown under high tunnels in Danish conditions

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Abstract

Seven raspberry cultivars were evaluated for berry yield, berry size and quality parameters over two consecutive seasons under tunnel conditions in conventional and organic fields in Denmark. There were field x cultivar, field x year and cultivar x year interactions in annual- and field x cultivar x year interaction in biannual-fruiter cultivars for yield. ‘Autumn Bliss’ produced the highest yield (4.6 kg m\textsuperscript{-1} row) from both organic and conventional field while ‘Glen Fyne’ yielded 11.1 kg m\textsuperscript{-1} row from organic field in 2013. ‘Autumn Treasure’ and ‘Octavia’ had the largest berries (4.1 and 5.8 g respectively). The peak period of harvesting of the annual-fruiter cultivars ranged from week no. 36 to 38, and week no. 30 to 33 for biannual-fruiter cultivars. There were field x cultivar, field x year and cultivar x year interactions in annual- and field x year and cultivar x year interactions in biannual-fruiter cultivars for total soluble solid (TSS). The highest TSS was found in ‘Fall Gold’ (9.7 °Brix) from organic field, and ‘Tulameen’ (11.5 °Brix). Field did not influence TSS and TSS: TA ratio in biannual-fruiter cultivars. A higher TSS, lower TA and higher TSS: TA ratio were found in 2013 compared to 2012 in biannual-fruiter cultivars. There were cultivar x field interaction for citric acid, and year x field interaction for malic acid in annual-fruiter cultivars. The highest citric acid content was observed in ‘Autumn Bliss’ (138.0 µg mg\textsuperscript{-1} DW) from conventional field. ‘Tulameen’ have the highest citric acid (178.0 µg mg\textsuperscript{-1} DW) and similar with ‘Octavia’. Sugars were not affected by fields but year variations were observed in annual-fruiter cultivars. The highest glucose (127.1 µg mg\textsuperscript{-1} DW) and fructose (146.3 µg mg\textsuperscript{-1} DW) were measured in 2012 but were not different between cultivars. Citric and malic acids were decreased in 2012 compared to 2011 whereas glucose and fructose were increased
in 2012 in annual-fruiting cultivars. Higher sucrose was measured in ‘Fall Gold’ and ‘Autumn Bliss’. Glucose and fructose were different between fields and a higher concentration was found in 2013 than 2012 in biannual-fruiting cultivars. ‘Tulameen’ has higher sucrose (130.4 µg mg⁻¹ DW) but similar with ‘Glen Ample’. From yield and quality analysis of all cultivars, ‘Autumn Bliss’ was the best cultivar for autumn production in organic field since it had high yield with large fruit and moderate TSS: TA ratio, and was consistent across the years and fields. ‘Octavia’ and ‘Glen Fyne’ are the promising cultivars for summer production in Danish conditions.

**Key Words:** Citric acid, Malic acid, Sugar, *Rubus idaeus*, Total soluble solids, Titratable acid

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1. Introduction

The consumption of raspberry products is encouraged worldwide because of an increase in consumer awareness on nutritional quality and health benefits (Rao and Snyder, 2010). As with other food commodities, there has been increased interest in consumption of raspberry fruits harvested from organic fields (Graham and Gennings, 2009). Organic food is gaining popularity because many consumers perceiving them as healthier and more nutritious foods and are willing to pay an additional 10% to 40% price premium (Winter and Davis, 2006). In additional to this, organic products are considered to have better sensory and long-term storage qualities but organic production has substantially much lower yields compared to conventional production (Rembialkowska, 2007).

Protected cropping in high tunnels and out-of-season production for fresh market across the European countries are increasingly expanding (Oliveira et al., 1996). High tunnels are adopted in raspberry production due to the potential to extend the cropping season and increasing overall fruit (Weber, 2010). Under tunnel production, raspberries are protected from rain and wind damage, reducing the prevalence of fungal diseases because of drier leaves and fruits (Pitsioudes et al., 2002; Carew et al., 2000). However, tunnels have the potential to reduce light intensity and increase air and soil temperatures which could negatively influence quality raspberry production in warm summer months. Also, quality aspects of raspberry fruits before and after harvest are depend on genetic and environmental factors such as light and temperature (Lister and Lancaster, 1996; Mori et al., 2005; Kassim et al., 2009). Content of sugars and organic acids vary with season and other growing conditions such as soil nutrient and water availability between organic and conventional field conditions (Etienne et al., 2013).

The effect of the post-flowering temperature (12, 18, and 24 °C) on fruit chemical composition of ‘Glen Ample’ were evaluated in controlled conditions and it was found that an increase in temperature resulted in reduced berry weight (both FW and DW basis) (Remberg et al., 2010). Moreover, the potential effect of temperature on fruit chemical composition is emphasized when application of protected cultivation is commercialized for out of season production (Pitsioudis et al., 2002; Sonsteby et al., 2009; Atkinson et al., 2005).

Although it is known that yield, concentrations of sugars and organic acids, and the sugar to acid ratio in raspberries are affected by growing conditions, cultivar and season, comparisons
have not been carried out for organic and conventional raspberries grown under high tunnel conditions in Denmark. In addition, little information on raspberry yield and quality is available in Denmark due to the recent introduction of many new cultivars. Therefore, we studied the effect of organic and conventional production, and post-flowering temperature inside tunnel on quality in three annual-fruiting and four biannual-fruiting cultivars. The aim was also to demonstrate the opportunities for sustainable organic production of raspberries of high quality in Danish conditions over two seasons.

2. Materials and methods

2.1 Experimental sites

Tunnel experiments were established to evaluate raspberry cultivars at Aarslev (10°27′E, 55°18′N), Aarhus University, Denmark in organic and conventional fields. The soil type was a sandy loam with a good to excessive drainage. Nitrate-N (18.2 mg kg⁻¹), P₂O₅ (3.1 mg 100⁻¹ g), K₂O (22.6 mg 100⁻¹ g) and Mg (4.2 mg 100⁻¹ g) were relatively higher in the conventional compared to organic field. In contrary, ammonium-N (2.6 mg kg⁻¹) and humus (3.1%) were higher in organic field.

2.2 Plant materials and growth conditions

One year old plants of annual-fruiting cultivars ‘Autumn Bliss’ (East Mailing, Great Britain), ‘Autumn Treasure’ (East Mailing, Great Britain) and ‘Fall Gold’ (East Mailing, Great Britain), and biannual-fruiting cultivars ‘Glen Ample’ (James Hutton Institute, Scotland), ‘Glen Fyne’ (James Hutton Institute, Scotland), ‘Octavia’ (East Mailing, Great Britain) and ‘Tulameen’ (Canada) were obtained from the nursery Vester Skovgaard, Denmark, and were planted on 19 April 2010 in a randomized complete block design with three replications in each field (organic and conventional). The plants used had previously been chilled at 2 ±1 °C for 10 weeks and were pruned down to soil level during planting. The distance between rows was 1.7 m with five plants per replication planted at 0.5 m intervals in the centre of 0.4 m wide raised beds and ten canes per meter row in both annual- and biannual-fruiting cultivars were maintained for yield and quality evaluation.

The system consisted of four rows in north-east direction. In the following year in April 2011, an open tunnel 3.6 m high, 8.5 m wide and 42 m long was built and covered with plastic (Atoplan Longlife film, Vorden, the Netherlands) film to protect fruits from rainfall.
Each winter (from November to April the following year), the plastic cover was removed. Biannual-fruiting cultivars were pruned at the height of 2 m each year before start of winter while annual-fruiting cultivars were pruned to soil level immediately after winter to remove the previous year’s growth and make room for the new primocanes.

2.3 Fertilizer application, irrigation, and plant protection

2.3.1 Organic field

The organic plot received a granular form of organic fertilizer ‘Binadan 5-2-4’ (5% total N, 1.8% total P and 3.8% water soluble K) (Binadan A/S, Frisbakvej 5, DK-8766, Denmark; www.binadan.dk) which was applied at the rate of 1500 kg ha⁻¹ (176 kg N ha⁻¹, i.e. 15 g plant⁻¹) in the establishment year (May, 2010). In the following years (2011, 2012 and 2013), 80 kg N ha⁻¹ was applied using the same fertilizer ‘Binadan 5-2-4’ in three splits; 50% at the end of March, 25% at the beginning of June and the remaining 25% at the third week of July. The ground was covered with black Mypex and plants were drip irrigated at the rate of 1 L day⁻¹ plant⁻¹. During the growing season, plants were cultivated, and pests and diseases were managed according to organically approved methods and Danish organic regulations. Phytoseiulus-System (Phytoseiulus persimilis) at 2000 predatory mites mixed with vermiculite (Biobest Belgium N.V., 2260 Westerlo, Belgium) (http://www.biobest.be) was used to control spider mites (Tetranychus urticae) under the tunnels. Two pheromone traps per tunnel were installed to control raspberry beetle (Byturus tomentosus) in April 2012. Spuzit (organic pesticide) (W. Neudorff GmbH KG An der Muehle 3 D-31860 Emmerthal, Germany) (http://www.neudorff.de/en/product-catalogue/spruzit-insecticide.html) (1%) was sprayed on 15 May 2012 and 2013 to control thrips, grubs, caterpillars, spider mites, beetle and sawfly larvae.

2.3.2 Conventional field

The conventional field was fertilized with 80:20:90 NPK kg ha⁻¹ in 2011 and 60:20:90 NPK kg ha⁻¹ in 2012 and 2013. Therefore, each plant got 9.5 g N in 2011 and 7.5 g N in 2012 and 2013, and 2.5 g P₂O₅ and 15 g K₂O (based on 8500 plants/ha). Magnesium was supplied at 15 kg Mg ha⁻¹ using MgNO₃ (10.7% N and 9.8% Mg) and MgSO₄ (9.8% Mg and 13% sulphur) in all three seasons. Fertilizers were mixed to provide nutrients through drip irrigation from May to September in 2011, May to August in 2012 and May to July in 2013. Phosphorus and
potassium were supplied by phosphoric acid 75 (23.7% P$_2$O$_5$) and potassium nitrate (15.5% N and 38.2% K$_2$O) respectively. The remaining of nitrogen was supplied by nitric acid (soluble, HNO$_3$) 62% (13.8% N) with irrigating water. Irrigation was provided using 2.4 L m$^{-1}$ drip capacity tubing at the rate of 2.4 L h$^{-1}$ m$^{-1}$ day$^{-1}$ for 50 min and therefore it was 1 L day$^{-1}$ plant$^{-1}$ (2 L m$^{-1}$ row day$^{-1}$). Spraying was done using Teldor (0.008%; dissolved in 200 L) on 21 May 2012 and 2013 as fungicide. Similarly dithane (0.2%) and perimor (0.05%; dissolved at 200 L) was sprayed in conventional field on 11 June 2012 and 2013.

2.4 Yield and berry size

Fruits were picked twice a week from week no. 28 to 36 each year for biannual-fruiting cultivars and week no. 32 to 44 for annual-fruiting cultivars. Berries were harvested from 10 canes (one m row) at a full ripe stage in both annual-and biannual-fruiting cultivars. The total marketable berry yield and berry size were recorded. Berry size was calculated by weighing the 20 representative fruits at each harvest.

2.5 Total soluble solid (TSS, °Brix) and titratable acid (%)

Fruit samples for analysis were collected at the 3rd and 4th weeks from the start of the harvesting season of year 2011, and 3rd and 5th weeks in 2012 and 3rd week in 2013. Harvested berries from week no. 36 and 38 for annual-fruiting cultivars in 2011 and 2012, and week no. 30 and 32 for biannual-fruiting cultivars in 2012 were stored at -20 °C for sugar and organic acid analysis. The fruit samples were defrozen, mixed with 1: 5 deionised water and stirred for 1 min in vertex. The mix was centrifuged at 4000 rpm and the supernatant was used to measure total soluble solid (°Brix) using a digital refractometer (Pocket PAL-1, Atago, Japan). Titratable acid (TA) was analysed using a digital biuret and 0.1 M NaOH to titrate samples to an endpoint of 8.1, and expressed based on % citric acid. The TSS and TA were calculated from the mean values of two technical replications over the mean of samples harvested at 3rd and 4th weeks in 2011, 3rd and 5th weeks in 2012 and 3rd week from the start of harvesting fruits in 2013.

2.6 Organic acids and sugar analysis

2.6.1 Organic acids and sugar extraction

Fifty gram of fresh fruit were collected at 3rd week of start of harvest period and stored at -20 °C before freeze dried. Samples were collected in 2011 and 2012 for annual-fruiting cultivars
but it was collected only in 2012 for biannual-fruiting cultivars. After lyophilized, samples were grounded into a fine powder which was subsequently vacuum packed and stored at 4°C until analysis.

To extract individual organic acids and sugars, 60 mg freeze dried material was weighted into a 2 mL eppendorf tube. Extraction was performed at room temperature for 30 min in 1.5 mL of extraction buffer (50: 49: 1, methanol: water: formic acid). The extracts were then centrifuged for 10 min (16000 x g, 1 °C) and 1 mL of the supernatant was collected into a separate tube. The tube was subsequently heated at 80 °C for 10 min and centrifuged for 10 min (16000 x g, 1 °C). Part of the supernatant (500 µL) was transferred into a separate tube and subsequently evaporated. Once evaporated, samples were re-suspended in 1 mL of deionised water.

2.6.2 Organic acids analysis

Two major organic acids (citric acid and malic acid) were quantified following extract dilution (1: 20) by anion exchange HPLC on a Dionex IonPac AS11-HC 4 x 250 mm column fitted with a 4 x 50 mm guard column according to a method based on that of Nwankno et al., (2012). Eluent A was 10% methanol in ultrapure water and eluent B was 100 mM NaOH containing 10% methanol. Eluent flow rate was 1.5 mL min⁻¹ and the gradient was as follows: 0 min, 10% B, 1 min, 10% B; 5 min, 25% B; 8 min, 30% B; 18 min, 60% B; 23 min, 80% B; 24 min, 10% B; 30 min, 10% B. Organic acids were detected by conductivity and ion suppression was undertaken at 200 mA using a 4 mm ASRS 300 in the external mode with ultrapure water at a flow of 2.0 mL min⁻¹. Organic acids were identified by co-elution with authentic standards of malic and citric acid and quantified by reference to appropriate standard curves.

2.6.3 Sugar analysis

Sugars (glucose, fructose and sucrose) were quantified following extract dilution (2: 1000) by anion exchange chromatography on a Dionex Carbopac PA-100 250 x 4 mm column. The mobile phase was 200 mM NaOH prepared in degassed water pumped at 1 mL min⁻¹ for 15 min in isocratic mode. Sugars were detected by pulsed amperometry using a standard quad waveform and quantified by reference to an authentic standard curve of each sucrose, glucose
and fructose. Sugar analysis of biannual-fruited cultivars was done in 2012 while it was done in 2011 and 2012 for annual-fruited cultivars.

2.7 Air temperature inside and outside tunnel

During the experimental period, pre- and post-flowering air temperatures, inside and outside the tunnel were recorded daily at weather stations over the three years (Figure 1). The pre-flowering months were warmer in 2012 and 2013 compared to 2011. A maximum temperature was 43.4 °C on 20 July 2012 inside organic tunnel and 36.1 °C on 24 June 2012 in conventional tunnel. The maximum temperature reached to 33.6 °C on 29 July 2011 inside organic tunnel while it was 31.5 °C on 5 August 2011 inside conventional tunnel. Similarly, maximum temperature was 34.5 °C on 2 August in 2013 inside organic tunnel but remained maximum temperature 31.9 °C on 26 July 2013 inside conventional tunnel. Similarly, the average temperature remained higher in 2012 and 2013 compared to 2011. Moreover, the average and maximum temperature inside organic tunnel remained higher across the season compared to conventional tunnel.
Figure 1. Maximum and average temperature of organic and conventional tunnels comparing inside and outside conditions across three years 2011, 2012 and 2013.

2.9 Statistical analysis

All data were subjected to statistical analysis using the General Linear Model (PROC GLM) of SAS (SAS Institute, Inc., 1999 - 2001, Cary, NC) with cultivar, field (organic and conventional) and year as class variables. Mean differences between and within cultivars, fields and years were separated using Tukey Kramer’s test at $P \leq 0.05$. The correlation coefficients ($r$) for TSS and TA analysed from the 3rd and 5th week after start of the berry
harvesting with the average of one week pre-harvest tunnel temperatures were calculated using temperature as independent variable.

3. Results

3.1 Yield

3.1.1 Annual-fruiting cultivars

There were significant field x cultivar, field x year, and cultivar x year interactions for yield in annual-fruiting cultivars (p<0.05) (Table 1). Yield was highest in ‘Autumn Bliss’ both in the organic and conventional field, and a similar yield was seen in ‘Autumn Treasure’ grown in the organic field. Within cultivars, field affected yield in ‘Autumn Treasure’ and a higher yield was found in the organic field (p<0.05) (Table 1; Figure 2). In contrary, there was no difference between fields in ‘Autumn Bliss’ and ‘Fall Gold’. In 2011 the mean yield of all cultivars was similar from the organic and conventional field whereas in 2012 there was a higher yield from the organic field. ‘Fall Gold’ produced the lowest yield (1.8 kg m⁻¹ row) in 2011. There was no difference between years for yield. ‘Fall Gold’ lower yield than ‘Autumn Bliss’ followed by ‘Autumn Treasure’.

Table 1. Berry yield (mean ± standard error) in three annual-fruiting raspberry cultivars from organic and conventional field in 2011 and 2012.

<table>
<thead>
<tr>
<th>Cultivar x field (n = 6)</th>
<th>Organic</th>
<th>Conventional</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Autumn Bliss’</td>
<td>4.6 ±0.17ᵃ</td>
<td>4.6 ±0.31ᵃ</td>
</tr>
<tr>
<td>‘Autumn Treasure’</td>
<td>4.3 ±0.33ᵃ</td>
<td>3.3 ±0.24ᵇ</td>
</tr>
<tr>
<td>‘Fall Gold’</td>
<td>2.2 ±0.32ᶜ</td>
<td>2.2 ±0.09ᶜ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year x field (n = 9)</th>
<th>Organic</th>
<th>Conventional</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>3.4 ±0.49ᵃᵇ</td>
<td>3.6 ±0.42ᵃᵇ</td>
</tr>
<tr>
<td>2012</td>
<td>4.0 ±0.35ᵃ</td>
<td>3.2 ±0.35ᵇ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cultivar x year (n = 6)</th>
<th>2011</th>
<th>2012</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Variety</th>
<th>2012 Yield</th>
<th>2013 Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Autumn Bliss’</td>
<td>4.5 ±0.22a</td>
<td>4.6 ±0.26a</td>
</tr>
<tr>
<td>‘Autumn Treasure’</td>
<td>4.1 ±0.31ab</td>
<td>3.5 ±0.36b</td>
</tr>
<tr>
<td>‘Fall Gold’</td>
<td>1.8 ±0.11c</td>
<td>2.6 ±0.20bc</td>
</tr>
</tbody>
</table>

Mean values followed by different lower-case letters within the interaction indicates a significant difference (P < 0.05) by Tukey’s test.

### 3.1.2 Biannual-fruiting cultivars

There was significant field x cultivar x year interaction for yield in biannual-fruiting cultivars (p<0.001). ‘Glen Fyne’ produced the highest yield (11.1 kg m⁻¹ row) from the organic field which was similar to ‘Glen Ample’, ‘Octavia’ and ‘Tulameen’ in organic field in 2013. The lowest yield was observed in ‘Tulameen’ in 2013 from conventional field. The highest yield was observed in ‘Glen Ample’ in 2013 (10.4 kg m⁻¹ row) and it had the lowest yield (4.3 kg m⁻¹ row) in 2012 from the conventional field (Figure 3). Yield was generally increased or similar in 2013 compared to 2012. In 2012, there was a similar yield in all cultivars whether grown in the organic or conventional field. In 2013, the yield in ‘Glen Fyne’ and ‘Tulameen’ was much higher from the organic field than the conventional field. In ‘Glen Ample’ and ‘Octavia’, no significant differences between fields were found.

### 3.2 Harvesting period

Berry harvesting of the annual-fruiting cultivars was started from week no. 33 in both 2011 and 2012 and continued until week no. 43 in 2011 and week no.44 in 2012 except in ‘Autumn Treasure’ (Figure 2). The peak period of the berry harvesting was week no.36 to 38 in both years for annual-fruiting cultivars.
Harvesting of biannual-fruitering cultivars ‘Glen Ample’, Glen Fyne’ and ‘Tulameen’ was started from week no. 28 and continued until week no. 36 in the organic field while in the conventional field, harvesting finished at week no. 35 (Figure 3). ‘Octavia’ was not harvested until week no. 30 in 2013 while in ‘Tulameen’ was harvested from week no. 29 in

Figure 2. Cumulative yield of annual-fruitering raspberry cultivars in 2011 and 2012. Bars on final point indicates standard error of mean of the total berry yield (n = 3).
the organic field in 2013. The peak period of berry harvesting for biannual-fruited cultivars was week no. 31 to 33 both years except for ‘Octavia’ and ‘Tulameen’ in 2013 in both organic and conventional fields (Figure 3).

![Graph showing cumulative yield of biannual-fruited raspberry cultivars in 2012 and 2013.](image)

**Figure 3.** Cumulative yield of biannual-fruited raspberry cultivars in 2012 and 2013. Bars on the final yield point indicate standard error of mean of the total berry yield (n = 3) and points followed by different lower-case letters indicate a significant difference (cultivar x field x year interactions) (P < 0.05) by Tukey’s test.
3.3 Berry size

Berry size differed between cultivars and fields (p<0.001) for annual-fruiting cultivars but year did not influence fruit size. ‘Autumn Treasure’ had the largest fruit (4.1 g) (data not shown). The smallest fruit (3.1 g) was observed in ‘Fall Gold’. The significantly largest berries (3.8 g) were observed from the conventional field while it was 3.4 g in organic field.

For biannual-fruiting cultivars, there were year x field and year x cultivar interactions (P<0.05). ‘Octavia’ had the largest berry (5.8 g) in 2012 while ‘Glen Fyne’ had the smallest berry (3.4 g) (Figure 4). On an average, ‘Glen Ample’ and ‘Octavia’ had the largest berries among four biannual-fruiting cultivars. Except in ‘Tulameen’ and ‘Glen Ample’, the berry size was bigger in 2012 than 2013.
Figure 4. Berry size (g) of biannual-fruited raspberry cultivars in 2012 and 2013. Bars indicate standard errors of mean (n = 6 for cultivar x year and n = 9 for field x year interactions). Mean values followed by different lower-case letters indicate a significant cultivar x year interactions (upper) and field x year (lower) (P < 0.05) by Tukey’s test.

3.4 Total soluble solids (TSS, °Brix), titratable acid (TA, %) and TSS: TA ratio

3.4.1 Annual-fruited cultivars
There were significant field x cultivar, field x year and cultivar x year interactions in annual-fruiting cultivars for TSS whereas titratable acid was significantly different between years and field x cultivar interaction for TSS: TA ratio (Table 3).

Table 3. Total soluble solid (TSS, °Brix), titratable acid (%) and TSS : TA ratio of three annual-fruiting and four biannual-fruiting raspberry cultivars from organic and conventional field in two years.

<table>
<thead>
<tr>
<th></th>
<th>TSS</th>
<th>TA</th>
<th>TSS:TA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Annual-fruiting cultivars</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic</td>
<td>9.0 a</td>
<td>1.9</td>
<td>4.9 a</td>
</tr>
<tr>
<td>Conventional</td>
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<td>2.0</td>
<td>4.5 b</td>
</tr>
<tr>
<td><strong>Cultivar</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Autumn Bliss’</td>
<td>8.6 b</td>
<td>1.9</td>
<td>4.7 ab</td>
</tr>
<tr>
<td>‘Autumn Treasure’</td>
<td>8.8 ab</td>
<td>2.1</td>
<td>4.5 b</td>
</tr>
<tr>
<td>‘Fall Gold’</td>
<td>9.1 a</td>
<td>2.0</td>
<td>4.9 a</td>
</tr>
<tr>
<td><strong>Year</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>8.6 b</td>
<td>2.4 a</td>
<td>3.5 b</td>
</tr>
<tr>
<td>2012</td>
<td>9.1 a</td>
<td>1.5 b</td>
<td>5.9 a</td>
</tr>
<tr>
<td><strong>Probability level by ANOVA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field (F)</td>
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<tr>
<td>Cultivar (C)</td>
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<tr>
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</tr>
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<td>ns</td>
</tr>
<tr>
<td>F x C x Y</td>
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<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Biannual-fruiting cultivars</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>Organic</td>
<td>9.3</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>---------------</td>
<td>---------</td>
<td>-----</td>
<td>----------------</td>
</tr>
<tr>
<td>Conventional</td>
<td>9.6</td>
<td>1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3</td>
</tr>
<tr>
<td><strong>Cultivar</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Glen ample’</td>
<td>8.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>‘Glen Fyne’</td>
<td>9.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>‘Octavia’</td>
<td>9.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>‘Tulameen’</td>
<td>10.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Year</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>8.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2013</td>
<td>10.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2&lt;sup&gt;a&lt;/sup&gt;</td>
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**Probability level by ANOVA**

<table>
<thead>
<tr>
<th>Field (F)</th>
<th>Cultivar (C)</th>
<th>Year (Y)</th>
<th>F x C</th>
<th>F x Y</th>
<th>C x Y</th>
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<td>0.0033</td>
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</tr>
</tbody>
</table>

ns, non-significant at P>0.05 by Tukey’s test

Mean values followed by different lower-case letters indicates a significant difference (P < 0.05) by Tukey’s test.

The significantly highest TSS was measured in ‘Fall Gold’ (9.7 °Brix) from organic field. The TSS was the higher in 2012 from the conventional field (Table 4). The highest TSS (9.3 °Brix) was found measured in ‘Autumn Treasure’ but similar with other cultivars in 2012. Higher TA (2.4%) was measured in 2011 whereas it was 1.5% in 2012. TSS: TA ratio was higher in organic field. Within cultivars, higher TSS: TA ratio was measured in ‘Fall Gold’
but was similar with ‘Autumn Bliss’ (4.7). TSS: TA ratio was found to be higher (5.9) in 2012.

Table 4. Total soluble solid (TSS) (mean ± standard error) in three annual-fruited raspberry cultivars from organic and conventional field in 2011 and 2012.

<table>
<thead>
<tr>
<th>Cultivar x field (n = 6)</th>
<th>Organic</th>
<th>Conventional</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Autumn Bliss’</td>
<td>8.7 ±0.11b</td>
<td>8.5 ±0.18b</td>
</tr>
<tr>
<td>‘Autumn Treasure’</td>
<td>8.7 ±0.24b</td>
<td>8.9 ±0.31b</td>
</tr>
<tr>
<td>‘Fall Gold’</td>
<td>9.7 ±0.19a</td>
<td>8.4 ±0.17b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year x field (n = 9)</th>
<th>Organic</th>
<th>Conventional</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>9.0 ±0.28a</td>
<td>8.2 ±0.10b</td>
</tr>
<tr>
<td>2012</td>
<td>9.1 ±0.14a</td>
<td>9.0 ±0.15a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cultivar x year (n = 6)</th>
<th>2011</th>
<th>2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Autumn Bliss’</td>
<td>8.4 ±0.18b</td>
<td>8.8 ±0.06b</td>
</tr>
<tr>
<td>‘Autumn Treasure’</td>
<td>8.4 ±0.13b</td>
<td>9.3 ±0.16a</td>
</tr>
<tr>
<td>‘Fall Gold’</td>
<td>9.1 ±0.43b</td>
<td>9.1 ±0.21a</td>
</tr>
</tbody>
</table>

Mean values followed by different lower-case letters within the interaction indicates a significant difference (P < 0.05) by Tukey’s test.

3.4.2 Biannual-fruited cultivars

There were field x year and cultivar x year interaction for TSS whereas TA and TSS: TA ratio were significantly different between cultivars and years in biannual-fruited cultivars (Table 3). The significantly highest TSS (11.5 °Brix) was measured in ‘Tulameen’ in 2013 which was similar with ‘Octavia’ (10.4 °Brix) (Table 5). There was higher TSS measured in 2013 compared to 2012 from each organic and conventional field. The highest TA (2.0%) was also found in ‘Tulameen’ and was higher in conventional field compared to organic field. On an average, TA was lower in 2013 compared to 2012. The highest TSS: TA ratio (6.1) was measured in ‘Glen Fyne’ and was also higher in 2013 (6.2) compared to 2012 (4.5).

Table 5. Total soluble solid (TSS) (mean ± standard error) in four biannual-fruited raspberry cultivars from organic and conventional field in 2012 and 2013.

<table>
<thead>
<tr>
<th>Cultivar x year (n = 6)</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Glen Ample’</td>
<td>8.3 ±0.38a</td>
<td>8.5 ±0.15a</td>
</tr>
</tbody>
</table>
Mean values followed by different lower-case letters within the interaction indicates a significant difference (P < 0.05) by Tukey’s test.

### 3.5 Organic acids

In annual-fruiting cultivars, citric acid was different between fields and cultivars but year did not affect it whereas malic acid was different between cultivars and years. There were also cultivar x field interaction for citric acid and year x field interaction for malic acid (Table 6).

<table>
<thead>
<tr>
<th>Field x year (n = 12)</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic</td>
<td>8.4 ±0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.1 ±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Conventional</td>
<td>9.1 ±0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.0 ±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 6. Organic acid (citric and malic acids, µg mg<sup>-1</sup> DW) and sugar (glucose, fructose and sucrose, µg mg<sup>-1</sup> DW) content of three annual-fruiting raspberry cultivars in 2011 and 2012 and four biannual-fruiting cultivars in 2012 from organic and conventional field.
<table>
<thead>
<tr>
<th>Field (F)</th>
<th>0.019</th>
<th>ns</th>
<th>ns</th>
<th>ns</th>
<th>ns</th>
<th>ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar (C)</td>
<td>0.0012</td>
<td>&lt;0.0001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.0001</td>
</tr>
<tr>
<td>Year (Y)</td>
<td>ns</td>
<td>&lt;0.0001</td>
<td>0.006</td>
<td>0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>F x C</td>
<td>0.038</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>F x Y</td>
<td>ns</td>
<td>0.018</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>C x Y</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
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<td>ns</td>
</tr>
<tr>
<td>F x C x Y</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Biannual-fruiting cultivars**

<table>
<thead>
<tr>
<th>Field</th>
<th>Organic</th>
<th>154.6</th>
<th>5.4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>109.3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>124.2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>93.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>155.7</td>
<td>4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>222.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102.1</td>
<td></td>
</tr>
</tbody>
</table>

**Cultivar**

- ‘Glen Ample’ | 134.3<sup>c</sup> | 4.5 | 121.2 | 133.1 | 118.4<sup>a</sup> |
- ‘Glen Fyne’ | 143.6<sup>bc</sup> | 4.4 | 113.1 | 137.1 | 79.5<sup>b</sup> |
- ‘Octavia’ | 164.8<sup>ab</sup> | 5.3 | 119.5 | 140.7 | 63.2<sup>b</sup> |
- ‘Tulameen’ | 178.0<sup>a</sup> | 5.0 | 109.5 | 115.5 | 130.4<sup>a</sup> |

**Probability by ANOVA for biannual-fruiting cultivars**

<table>
<thead>
<tr>
<th>Field (F)</th>
<th>ns</th>
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<th>0.043</th>
<th>0.046</th>
<th>ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar (C)</td>
<td>0.0003</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.0001</td>
</tr>
<tr>
<td>F x C</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

The highest citric acid was measured in ‘Autumn Bliss’ (138.0 µg mg<sup>-1</sup> DW) from conventional field which was similar with other cultivars and fields except in ‘Fall Gold’ (108.7 µg mg<sup>-1</sup> DW) from organic field (Table 7). Similarly, malic acid was higher in 2011 (4.2 µg mg<sup>-1</sup> DW) from conventional field.

**Table 7.** Citric and malic acid content (µg mg<sup>-1</sup> DW) of three annual-fruiting raspberry cultivars from organic and conventional field in 2011 and 2012.
In biannual-fruited cultivars, citric acid was different between cultivars whereas malic acid was different between years (Table 6). The highest citric acid (178.0 µg mg⁻¹ DW) was measured in ‘Tulameen’ and ‘Glen Ample’ has the lowest citric acid (134.3 µg mg⁻¹ DW). Malic acid was the highest (5.4 µg mg⁻¹ DW) from organic field while it was 4.2 µg mg⁻¹ DW from conventional field.

### 3.6 Sugars

Glucose and fructose were different between years whereas sucrose was different between cultivars in annual-fruited cultivars (Table 6). Glucose (127.1 µg mg⁻¹ DW) and fructose (146.3 µg mg⁻¹ DW) concentration were found to be the highest in 2012 compared to 2011. The highest sucrose (140.7 µg mg⁻¹ DW) was measured in ‘Fall Gold’. On an average, glucose, fructose and sucrose were higher in 2012 than 2011 and fructose and sucrose were higher in organic field than conventional field in annual-fruited cultivars.

In biannual-fruited cultivars, glucose and fructose were different between fields whereas sucrose was different between cultivars. Glucose was 222.4 µg mg⁻¹ DW while fructose was 129.0 7 µg mg⁻¹ DW from conventional field. The highest sucrose (130.4 µg mg⁻¹ DW) was measured in ‘Tulameen’. On an average, glucose, fructose and sucrose were higher from conventional field in biannual-fruited cultivars.

### 3.7 Correlation coefficient (r)

#### 3.7.1 Correlation coefficient (r) for TSS and TA with preharvest tunnel temperatures
The TSS and TA were analysed for their functional relationship with the average tunnel temperature preceding harvest (Table 8). The cultivar responses to temperature for TSS and TA were not consistent across the years and fields. The TSS for ‘Autumn Bliss’ showed a negative correlation with preharvest temperature ($r = -0.79$) in organic field in 2012 but it was poor negative correlation with temperature in both years in the conventional field. Similarly, ‘Autumn Treasure’ had strong negative correlation in 2011 in the conventional ($r = -0.78$) and organic fields ($r = 0.60$). In contrary, correlation coefficient ($r$) with preharvest temperature in ‘Autumn Treasure’ and ‘Fall Gold’ in 2012 showed positive correlation ($r = 0.65$ and $0.73$ respectively) in 2012. ‘Glen Ample’ and ‘Octavia’ showed strong negative correlation for TSS with temperature in 2012 both in the organic and the conventional fields.

**Table 8.** Correlation coefficient ($r$) for TSS and TA with one week average and pre-harvest tunnel temperature of 2011, 2012 and 2013.

<table>
<thead>
<tr>
<th>Fields</th>
<th>Cultivars</th>
<th>$r$ for TSS</th>
<th>$r$ for TA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2011</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional</td>
<td>‘Autumn Bliss’</td>
<td>-0.12</td>
<td>-0.74</td>
</tr>
<tr>
<td></td>
<td>‘Autumn Treasure’</td>
<td>-0.78</td>
<td>-0.64</td>
</tr>
<tr>
<td></td>
<td>‘Fall Gold’</td>
<td>-0.41</td>
<td>0.32</td>
</tr>
<tr>
<td>Organic</td>
<td>‘Autumn Bliss’</td>
<td>-0.32</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>‘Autumn Treasure’</td>
<td>-0.60</td>
<td>-0.22</td>
</tr>
<tr>
<td></td>
<td>‘Fall Gold’</td>
<td>-0.43</td>
<td>-0.80</td>
</tr>
<tr>
<td><strong>2012</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional</td>
<td>‘Autumn Bliss’</td>
<td>-0.25</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>‘Autumn Treasure’</td>
<td>0.65</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>‘Fall Gold’</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>‘Glen Ample’</td>
<td>-0.17</td>
<td>-0.78</td>
</tr>
<tr>
<td></td>
<td>‘Glen Fyne’</td>
<td>0.26</td>
<td>-0.99</td>
</tr>
<tr>
<td></td>
<td>‘Octavia’</td>
<td>-0.98</td>
<td>-0.95</td>
</tr>
<tr>
<td></td>
<td>‘Tulameen’</td>
<td>-0.51</td>
<td>-0.86</td>
</tr>
<tr>
<td>Organic field</td>
<td>‘Autumn Bliss’</td>
<td>-0.79</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>‘Autumn Treasure’</td>
<td>-0.12</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>‘Fall Gold’</td>
<td>0.29</td>
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</tr>
<tr>
<td></td>
<td>‘Glen Ample’</td>
<td>0.06</td>
<td>-0.69</td>
</tr>
<tr>
<td></td>
<td>‘Glen Fyne’</td>
<td>0.49</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>‘Octavia’</td>
<td>-0.36</td>
<td>-0.25</td>
</tr>
<tr>
<td></td>
<td>‘Tulameen’</td>
<td>0.80</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>2013</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Discussion

4.1 Berry yield and size

In our tunnel experiments over two years, cultivars showed differences in the potential yield but interacted with years and fields. Although berry size of ‘Autumn Treasure’ was higher, yield was lower to ‘Autumn Bliss’ which could be due to low number of fruits per harvest (data not shown). ‘Fall Gold’ yielded only 1.8 kg m$^{-1}$ row in 2011. The poor yield of ‘Fall Gold’ in our study could be due to low numbers of fruiting laterals (data not shown). However, full crop potential of ‘Fall Gold’ was not realized in 2011 in ‘Fall Gold’ due to its late bearing habit.

The yield in biannual-fruitting cultivars unexpectedly higher from organic field except ‘Glen Ample’ and was constantly higher in 2013. For example, ‘Glen Fyne’ produced the highest yield (11.0 kg m$^{-1}$ row) in organic field in 2013 which was more than 2 times higher compared to conventional field in the same year. The reason for surprisingly higher yields from organic field was unknown to our knowledge. On the other hand, ‘Tulameen’ produced the lowest yield (2.5 kg m$^{-1}$ row) in conventional field in 2013 which was four times lower than organic field. The reduced yield in ‘Tulameen’ in conventional field could be due to the powdery mildew infection in leaves and fruits during flowering, fruiting and harvesting period (data not shown). However, possible control means (see materials and methods) of the fungus was carried out, but yield was reduced. This indicated that there is a potential differences in disease resistant between raspberry cultivars. However, we did not determine the disease scores in the fields.

Sonsteby et al., (2009) reported that high yield was associated with cane height, the number and length of laterals, and a low proportion of dormant buds in ‘Glen Ample’. It has also been reported that the single, most important component was the length of the flowering laterals, which accounted for 82% of the yield variation. In our experiements, canes in annual-fruiting cultivars were pruned down to soil level during winter, whereas biannual-fruiting cultivars were pruned at 2 m height, and we did not determine the cane height and no. of canes per plant.
Berry size is one of the selection criteria in breeding for high yield in raspberries (Stephens et al., 2009) because berry size is an important yield component. It has also been reported that the larger the berry size, the higher the water content and therefore, lower the dry matter. In our experiments, ‘Autumn Treasure’ had the largest berry (4.1 g) among three annual-fruiting cultivars but yield was lower than ‘Autumn Bliss’. Berry size was also influenced by field conditions, e.g. larger berries were obtained from conventional field in annual-fruiting cultivars. Berry size was significantly reduced with increased in the growth temperature and progress of the harvest season (Remberg et al., 2010). It was found that berry weight decreased with increasing temperature from 12 to 24 °C. Prive et al., (1993) reported that berry count is a more important determinant of yield than berry weight. Fruit maturation and harvest were advanced in increasing post-flowering temperature and revealed a yield potentialily of 2.5 kg per plant in ‘Glen Ample’ (Remberg et al., 2010). Berry size was apparently influenced by field in annual-fruiting cultivars compared with biannual-fruiting cultivars. This could be the genetic factor.

4.2 Berry quality

Berry quality is a degree of superiority, taste and the perception of conditions acceptable to consumers. These perceptions are the attributes which give value to the berry as evaluated by the consumer. The overall consumer appreciation is related more to the TSS: TA ratio than to the TSS content alone. The concentration of main soluble sugars and citric acid equivalent continue to increase with ripening the fruit, leading to a higher TSS: TA ratio. Sugar: acid ratio contributes characteristic taste of a fruit and so is an indicator of commercial and organoleptic ripeness. During the ripening process, acids are degraded, the soluble solid and sugar content increase and the sugar: acid ratio is increased. The balance between the sugars and acids has special important for consumer acceptability. Therefore the compositions and contents of organic acids and sugars are important factors influencing the organoleptic properties of fruits and juice.

Total soluble solids, titratatble acid, sugar and organic acid content are the major berry quality attributes for table purposes. There were cultivar x field, year x field and cultivar x year interaction in annual-fruiting cultivars, and field x year and cultivar x year interactions in biannual-fruiting cultivars for TSS. It indicates that TSS depends on both genetic and environmental factors and neither single factor is responsible for TSS variation in the
raspberry cultivars. The variations in tunnel temperature between fields and years may also influence TSS content. A study in a controlled condition found that the TSS and TA were enhanced by increasing temperature during fruit development from 12 to 24 °C (Remberg et al., 2010). The correlation coefficient for TSS and TA with average of one week pre-harvest tunnel temperatures showed that TSS was negatively correlated in most of the raspberry cultivars. More TSS and less TA indicate sweeter fruits and found in 2013 than 2012 in the biannual-fruitering cultivars. In the annual-fruitering cultivars, fruit was generally sweeter in 2012 than 2011, and organically grown ‘Autumn Bliss’ and ‘Fall Gold’ were sweeter than conventional grown fruits.

Quantification was performed for two major organic acids (citric and malic acid) in this experiment over two seasons for annual cultivars and one season in biannual-fruitering cultivars. Citric acid is major organic acid of the soluble fraction with low level of malic acid in raspberry (Wang et al., 2009). Citric acid and malic acid were present at high levels when the fruit were at the immature stage and acid level decreased with increasing maturity and fruit weight (Perkins-Veazie and Nonnecke, 1992; Wang et al., 2009). With the progress of the fruit maturation and harvest season, TSS, TA and dry matter increased (Remberg et al. 2010). It has also been reported that soluble solids and TA were enhanced by increasing temperature from 12 to 24 °C in controlled conditions which was contrary to our finding. While in strawberry, Wang and Camp (2000) found that increasing growth temperatures decreased fruit quality including soluble solids (TSS), titratable acid (TA), TSS: TA ratio which agrees with our finding (Table 8).

In general, fruits contained lower sucrose than fructose and glucose. The low sucrose content in the fruit is probably due to enzymatic hydrolysis after translocation from the leaves (Wang et al., 2009). Since fructose is characteristically sweeter than glucose or sucrose, its concentration is a desirable organoleptic trait. With fruit maturation and ripening in raspberry, there is an increase in fruit weight and sugar levels but decreases in organic and titratable acid (Perkins-Veazie and Nonnecke, 1992). In our study, glucose, fructose and sucrose were different between years in ‘Autumn Bliss’ and ‘Autumn Treasure’. There was field x year interactions for TSS: TA ratio in ‘Autumn Bliss’ which indicated that the TSS: TA ratio depended both on field and years. Sucrose content in the fruit is low and is probably due to enzymatic hydrolysis after translocation from the leaves (Wang et al., 2009). The effect of location in total soluble solid has been reported in other studies (Burrows and
Moore, 2002; Bradish et al., 2012) which might be due to temperature and cultivar difference. It has also been reported that plants grown at 18/12 °C greatest amounts of fructose, glucose, and total carbohydrates in fruit and decreased with increasing temperature while the greatest sucrose was found at 25/12 °C and the lowest was at 30/22 °C (Wang and Camp, 2000). The concentration of glucose, fructose and sucrose may also vary and depend on the time of the day of sampling e.g. sunny day or shady. In our experiment, solar radiation of the fruit sampling day for sugar analysis was varied between years and genotypes (e.g. annual- or biannual-fruiting cultivars).

Studies reported that higher content of total sugars in organically produced vegetables and fruits such as carrots, sugar beet, red beetroot, potatoes, spinach, Savoy cabbage, cherries, redcurrants, apples etc. (Rembialkowska, 2007). When nitrogen is available easily and abundantly, plant synthesises nitrogen containing compounds like proteins and nitrogen-containing secondary metabolites such as alkaloids and glucosinolates but when nitrogen is limited, the metabolism changes more towards carbon containing compounds like starch, cellulose and non-nitrogen containing secondary metabolites such as phenolics and terpenoids (Brandt and Molgaard, 2001). Berries with pleasant sensory characteristics often have high sugars and relatively low acids (Del Castillo et al., 2004; Tiitinen et al., 2005). Fructose and glucose were positively correlated with total soluble solids content in the fruit (Wang et al., 2009). In general, fruits contain lower sucrose than fructose and glucose. The low sucrose content in the fruit is probably due to enzymatic hydrolysis after translocation from the leaves.

5. Conclusion
Growing raspberries in a high tunnel with annual- and biannual-fruiting cultivars showed a possibility to produce fresh and quality organic raspberries during summer and autumn months under Danish conditions. Tunnel production also provides the opportunity for biological control of pest and diseases. Cultivar ‘Glen Fyne’ and ‘Octavia’ can be recommended for organic production for summer months as these produce high yield. ‘Autumn Bliss’ can be recommended for autumn production as it produces high yield with large fruits and a high TSS: TA ratio every year. However, ‘Fall Gold’ showed higher TSS content but due to low yield it cannot be recommended for commercial production.
References


Chapter 12: General discussion

The present work is based on six hypotheses concerning the effects of temperature on photosynthesis, chlorophyll pigments, flowering behaviours, gene expression and fruit yield and quality of commercial raspberry cultivars (Chapter 1, Thesis). Two main experiments were set up to test the hypotheses (Chapter 8, Thesis) and results are discussed in Papers I and II and Manuscript III (Chapters 9, 10 and 11, Thesis). However, the answers to the postulated hypotheses, implications of the results and shortcomings of the methods used in the experiments have not been illustrated in details. Therefore in this present discussion, an overview of results and previous works, and links with present science and knowledge are discussed. New knowledge and science gained from the three related experiments are also summed up.

12.1 Hypothesis I: High temperature stress decreases chlorophyll fluorescence

12.1.1 Heat stress and chlorophyll fluorescence

Based on the results shown in Figures 1 and 2 in Paper I, this hypothesis was accepted because all five cultivars showed a decrease in variable chlorophyll fluorescence (Fv/Fm) during a seven day exposure at 27, 32 and 37 °C across all cold stored plants. The highest reduction in Fv/Fm reached 0.70 after seven days at 37 °C in ‘Autumn Bliss’ and ‘Fall Gold’ (Figure 1, Table 1, Paper I) while it decreased to 0.72 at 37 °C in ‘Autumn Treasure’ and ‘Erika’ and was comparable to reference plants grown under greenhouse conditions (Fv/Fm, 0.80 - 0.82). There was a faster reduction in chlorophyll fluorescence from day 1 to 5 than from day 6 to 7 during exposure to heat stress. When comparing our results with other novel works in raspberries, but in field and potted conditions (e.g. Molina-Bravo et al., 2011), the heat sensitive raspberry cultivars ‘Qualicum’ had lowest Fv/Fm (0.43) after 40 minutes at 45 °C and in ‘Latham’ it was 0.64. It has been proposed that raspberry plants that have an average Fv/Fm below 0.60 after a heat shock at 45 °C for 40 min are considered heat susceptible, and conversely, those with values above 0.60 are heat tolerant. However, results in this study cannot be compared directly with the study by Molina-Bravo et al., (2011), we can speculate that cultivars used in our experiment were not highly susceptible to heat stress up to 37 °C for a seven day. Heat stress effects on the PSII photo-oxidizing site thereby decreases variable chlorophyll fluorescence (Georgieva et al., 2000; Ashraf and Harris, 2013). Sustained depression in Fv/Fm could be due to decrease in photochemistry (Figure 5.1,
Chapter 5; Thesis) or photoprotective energy dissipation (Adams III and Demming-Adams, 2004). Evidence from the literature suggests that CO₂ uptake and photosynthetic yield are reduced at high temperature stress (Zhang et al., 2001). However, a decrease in Fv/Fm suggests that the plants were experiencing stress but the nature of the stress and the underlying mechanism responsible for the depression in PSII efficiency requires additional investigation to make reliable conclusion according to Adams III and Demming-Adams, (2004). Factors other than heat stress may have influenced Fv/Fm in our experiment. For example, additional stress due to reduced water availability creating mild water stress in the afternoon and sink limiting conditions due to potted conditions with insufficient rooting volume (3.5 L pot) may be confounded with the reduction in Fv/Fm. Water stress is known to modify the response to heat stress (Lu and Zhang, 1999). In contrast to this, moderate water stress was not a limitation in other studies as moderate water availability helped to maintain a high PSII efficiency and prevented photoinhibition in grapevines (Flexas et al., 1998). Previous studies have also reported that the maximum quantum yield of PSII photochemistry measured by Fv/Fm was not affected by drought stress in strawberry (Razavi et al, 2008) and wheat (Lu and Zhang, 1999). Only a certain degree of photoinhibition can occur under extreme drought conditions (Flexas et al., 1999).

Moreover, the computed value of Fv/Fm from fluorescence in control conditions (e.g. greenhouse/climate chambers) is typically lower than in the open field due to differences in light (Adams III and Demming-Adams, 2004) which may apply in our study too. We carried out this experiment in climate chambers with irradiance constant at 350 µmol m⁻² s⁻¹ PAR while under field conditions, PAR may reach up to 1500 µmol m⁻² s⁻¹ on a bright sunny day in Danish conditions. Therefore our result might need verification under field conditions which is outside the scope of this thesis work.

12.1.2 Chlorophyll fluorescence and diurnal variations

In our experiment, Fv/Fm for ‘Polka’ was always higher in the morning and evening compared to midday values regardless of temperature regimes, heat stress treatment and cold storage duration. We found that the midday Fv/Fm decreased with an increased stress period and increased temperature across all cold store treatments (Figure 2; Paper I). The Fv/Fm was reduced from 0.82 to 0.71 after 7 days of treatment at 37 °C at midday but increased up to 0.77 in the evening and remained above 0.79 in the morning during the whole treatment period (Figure 7). The depression at midday and the partial recovery in the evening indicated
that photoinhibition could be partially reversible in raspberries. Moreover, results indicate that $F_v/F_m$ did not fully recover in the morning as $F_v/F_m$ did not regain 0.82 in any evaluation days after consecutive stress periods, thus the repair mechanism was not complete. This could also be due to increased water availability since we irrigated the plants before the $F_v/F_m$ was measured in the evening. Another reason behind the midday depression could also be explained by the effect of vapour pressure deficit (VPD). The VPD is a function of temperature and increases progressively with increasing temperature. Keeping a constant 60% RH affected VPD. In our experiment, reference plants were grown at 20 °C and 60% RH, thus the VPD was 0.94 kPa while it was 1.47, 1.90 and 2.50 kPa VPD for the plants grown at 27, 32 and 37 °C respectively. The stomatal conductance ($g_s$) may decrease directly as VPD increases and or $g_s$ may decrease as VPD increases because of an increase in water loss from the inside leaf to the outside air, lowering the leaf water potential (Streck, 2003). We can conclude there must have been increased transpiration with increasing temperature and an increasing level of temperature regimes at relatively constant 60% RH during the experimental period due to leaf-to-air VPD in our experiment. High VPD > 3 kPa caused reductions in Rubisco activity and affected carboxylation efficiency in *Prosopis juliflora* but the relative quantum yield of PSII and electron transport rates were not affected at increased VPD levels (Shirke and Pathre, 2004). There could be low soil water status confounding with the stomatal response to VPD because we irrigated the plants to pot capacity (3 L pot) in the morning, and before evening measurements. However $F_v/F_m$ was not affected by mild drought stress in other studies (Flexas et al., 1998; Lu and Zhang, 1999; Razavi et al, 2008).

The evening measurement of $F_v/F_m$ was taken after watering at 5.00 PM which might help to increase $F_v/F_m$ in the evening. Molina-Bravo et al., (2011) and Sharma et al., (2012) also reported diurnal variation of $F_v/F_m$ in field grown raspberry and greenhouse grown wheat respectively but increased $F_v/F_m$ in the evening in these studies was more likely due to reduced light intensity. In our study, the light intensity remained constant. Therefore, our findings imply that adaptation to heat stress in raspberry cultivars occurs by temporarily interconverting the PSII heterogeneity up to 37 °C which was in accordance with the finding of Mathur et al. (2011) in wheat. Adams III and Demmig-Adams (2004) proposed a sustained (zeaxanthin + antheraxanthin)-dependent thermal energy dissipation involved in sustained $F_v/F_m$ depression. However, we could not verify the thermal energy dissipation in this experiment which is outside the scope of this thesis.
12.1.3 Chlorophyll fluorescence and cold storage period

The F/\text{F}_m decreased in a similar pattern during the period of increased temperature for plants cold stored for 15 and 16 weeks but for plants cold stored for 17 weeks, F/\text{F}_m dropped quickly. There was a relatively faster drop in F/\text{F}_m at 32 and 37 °C in all cultivars and cold store period until day five compared to day six to seven (Figure 1, Paper I). The significantly stronger depression of F/\text{F}_m in plants cold stored for 17 weeks, as compared to 15 and 16 weeks may imply the operation of a quantitative chilling effect but this could not be proven by the results of the present study. There could also be other reasons for the differences in response in plants from the different cold store periods such as the light and temperature conditions during the initial growing period under greenhouse conditions.

In conclusion, the dark-adapted value of F/\text{F}_m could be an indicator for screening high temperature resistant cultivars in perennial species with maximum values around 0.83 for most plant species at optimal conditions. Statistical analysis showed that cold storage duration (C) x temperature (T) x stress period (S) x time of the day (D) and C x T x D interaction were not significant while C x T x S and T x S x D were significant for F/\text{F}_m. This implies that no single factor (cold store duration, temperature treatment, stress period or time of day) was alone responsible in decreasing F/\text{F}_m. All the cultivars used in the experiment showed decreased F/\text{F}_m over the period of seven days at 27, 32 and 37 °C but this did not clearly differentiate the cultivars in categories of more or less heat susceptible or tolerant ones. It is therefore necessary to examine the cultivars under additional periods of temperature stress, e. g. above 37 °C for longer than seven days in order to identify and screen clearly heat tolerant cultivars. This was also outside the scope of this thesis work.

12.2 Hypothesis II: High temperature stress decreases chlorophyll pigments in raspberries

The content of pigments in the leaves is also an important variable to evaluate the photosynthetic efficiency of plants. A number of studies in the literature report that heat stress affects the concentration and ratios of chlorophyll pigments and their composition depending on the thermotolerance capacity of the plant species (Camejo et al., 2005; Guo et al., 2006; Efeoglu and Terzioglu, 2009). In our experiment, we observed that chlorophyll a and chlorophyll a/b decreased at 37 °C in ‘Autumn Bliss’, ‘Fall Gold’ and ‘Polka’ compared to reference plants in greenhouse conditions whereas there was no effect in ‘Autumn Treasure’. The decrease in chlorophyll a at 37 °C was 58% in ‘Autumn Bliss’ and 47% in
‘Fall Gold’ (Figure 3A; Paper I). ‘Autumn Bliss’ had 5.9 mg g⁻¹ DW chlorophyll a in reference plants while it was 2.6 mg g⁻¹ DW at 37 °C. The lowest chlorophyll a (2.5 mg g⁻¹ DW) and chlorophyll b (1.1 mg g⁻¹ DW) was observed in ‘Fall Gold’ at 37 °C. ‘Autumn Treasure’ and ‘Erika’ had the highest chlorophyll a (5.5 and 5.2 mg g⁻¹ DW respectively) at 37 °C (data not shown). The chlorophyll a/b was lowest in ‘Polka’ at 37 °C. Chlorophyll a/b is an indicator of the functional pigment and light adaptation/acclimation capacity of the photosynthetic apparatus (Camejo et al., 2005).

Low chlorophyll a content suggests a decrease in light harvesting capacity (Adams and Barker, 1998). A previous study has reported that chlorophyll a and chlorophyll b are degraded at high temperature stress (Karim et al., 1999). Low accumulation of chlorophyll at high temperature may be attributed to impaired chlorophyll synthesis and or its degradation. The impaired chlorophyll biosynthesis is due to the destruction of enzymes for biosynthesis (Reda and Mandoura, 2011). For example, the activity of 5-aminolevulinate dehydratase (ALAD) decreased in cucumber under high temperature regimes (Mohanty et al., 2006). Tewari and Tripathy, (1998) found that chlorophyll synthesis at 42 °C in cucumber reduced by 60%. It has also been reported that reductions in photosynthetic pigments disturb the electron transport and hence reduce photosynthetic efficiency. The reduction in the electron transport system (ETS) was not verified in this thesis work but our results in Figure 1 and Table 1 of Paper I proved that photosynthetic efficiency was decreased at an elevated temperature regime which was further confirmed by the results presented in Table 12.1.

In our study, the ratio of chlorophyll (a+b)/carotenoid (x+c) was significantly different between temperature regimes only in ‘Erika’ where it ranged from 5.92 to 4.95. However, other cultivars also had a ratio above 5.0, but were not significantly different from reference plants. Carotenoids are necessary for photoprotection of photosynthesis and they play an important role as a precursor in signalling under abiotic stress. The carotenoid pigments in leaves are xanthophylls and they play an important role in light harvesting and photoprotection (Misra et al., 2006). The xanthophyll cycle (the reversible inter-conversion of two particular carotenoids, violaxanthin and zeaxanthin) is involved in photo-protection and xanthophyll carotenoids have been shown to stabilize and photo-protect the lipid phase of the thylakoid membranes (Sharkey, 2005). In high temperature stress conditions, the xanthophylls, including violaxanthin, antheraxanthin and zeaxanthin, are partitioned between the light harvesting complexes and lipid phase of the thylakoid membranes (Adams and
Demmig-Adams, 1992). As we have discussed in section 12.1.2, there could have been mild water stress in the potted raspberry plants which may have had an additive effect in decreasing chlorophyll pigment in the leaf. Previous studies reported that content of chlorophyll a and b changes under drought stress (Farooq et al., 2009) because drought stress inhibits chlorophyll a/b synthesis and decreases the content of chlorophyll a/b binding proteins, leading to reduction of the light-harvesting pigment protein associated with photosystem II (Sayed, 2003), but in this study, we were not able to reach any conclusion about the role of the additional effect of mild water stress in decreasing chlorophyll pigments.

In summary, the measured levels of chlorophyll a and chlorophyll a/b indicate that ‘Autumn Bliss’ and ‘Fall Gold’ are less heat tolerant than ‘Autumn Treasure’ and ‘Erika’. The high ratio of chlorophyll (a+b)/carotenoid (x+c) (i.e. above 5.0) in all cultivars across the temperature regimes and cold storage periods imply that photo-protection occurred at heat stress conditions in annual-fruited raspberries.

12.3 Hypothesis III: High temperature stress affects subsequent growth and flowering behaviour

12.3.1 High temperature stress and flowering behaviour

Flowering in annual-fruited raspberries is dependent on the temperature and photoperiod as shown by detailed studies by Carew et al., (2000, 2003) and Sonsteby and Heide (2009, 2010, 2012). In contrast to the previous work, we focused on the effects of elevated temperature regimes for a seven day period during early flower initiation on flowering and fruiting behaviours of annual-fruited cultivars. Moreover, treatments were performed in growth chambers and plants thereafter grown in high tunnels. We hypothesised that high temperature above 20 °C inhibits flower induction whereas flower development is advanced. For example, Carew et al., (2000, 2003) reported that time to flowering was shortest (110 days) at 22 °C in ‘Autumn Bliss’ while it was 160 days at 13 °C during the growth period. Time to anthesis decreases with increased temperature up to 24 °C. In our experiment, the number of days to anthesis of the terminal flower did not change in any of the five cultivars after a seven day period in elevated temperature regimes before transfer into open tunnel conditions at the Research Centre, Aarslev, Denmark (Figure 4, Paper I), but the percentage of unopened flower buds decreased in ‘Autumn Treasure’ (22%) and ‘Erika’ (18%) by a seven day period at 37 °C compared to reference plants.
After exposure to high temperature (37 °C), anthesis of the terminal flower in ‘Autumn Bliss’, ‘Fall Gold’ and ‘Polka’ was earlier while in ‘Autumn Treasure’ and ‘Erika’, it tended to be delayed numerically by 3 - 8 days compared to reference plants at 20 °C but was not statistically different. The reason that no significant differences were found could be the short stress period (seven days) imposed at flower initiation time before transfer into open tunnel conditions. Our finding was in agreement with Sonsteby and Heide, (2010) who also observed that ‘Autumn Treasure’ delays flowering above 20 °C but plants received above 20 °C for more than five weeks.

The number of unopened axillary buds at the main shoot decreased when ‘Autumn Bliss’, ‘Autumn Treasure’, ‘Fall Gold’ and ‘Polka’ were grown at 37 °C whereas the number of lateral shoots per plant increased in ‘Autumn Bliss’ (175%), ‘Erika’ (66%) and ‘Fall Gold’ (31%) compared to reference plants at 20 °C (Table 2, Paper I). The percentage of flowering lateral shoots per plant decreased by 16% in ‘Autumn Bliss’ after exposure to 37 °C for seven days but other cultivars were not affected by the temperature treatments. The literature suggests that in order to increase flowering laterals and early fruits, annual-fruiting cultivars need to be grown at 20 - 25 °C during the season. Advanced flowering, fruiting and ripening with higher yield was also reported in ‘Erika’ and ‘Polka’ growing in a range of temperature of 15 - 25 °C (Sonsteby and Heide, 2012). Moreover, the literature reports that elevated temperature regimes have a dual effect on flowering in annual-fruiting raspberries, for example, earliness in flowering at higher temperature regimes and extension of the flowering period (Heide et al., 2011). This was partially confirmed by our present results. For example, in our observation, day-to-first flower opening in ‘Autumn Bliss’, ‘Fall Gold’ and ‘Polka’ was earlier numerically but not significantly when plants were exposed at temperatures higher than 20 °C for a seven day at floral initiation period. ‘Autumn Bliss’ showed advance in flowering by up to 9 days. Exposure to temperature above 27 °C for a seven day enhanced laterals while the shoot growth and number of nodes after stress period decreased in almost all cultivars. Similarly, the percent of flowering laterals decreased by 16% in ‘Autumn Bliss’ when exposed at 37 °C for a seven day at floral initiation stage. The percentage of unopened flower buds per plant decreased in ‘Autumn Treasure’ and ‘Erika’ up to 42% compared to reference plants. On the other hand, the number of dormant buds decreased at 37 °C. In contrast to ‘Autumn Bliss’, flowering was delayed in ‘Autumn Treasure’ and ‘Erika’. We did
not examine and determine the effect of elevated temperature regimes on extension to the flowering period in any of these cultivars in this thesis work.

12.3.2 Cold store duration and flowering behaviours
Raspberry cultivars responded to cold treatment differently. In our study, there was a higher number of unopened axillary buds, a higher percent of flowering lateral shoots per plant and a higher number of flowers and buds per lateral in ‘Autumn Bliss’ and ‘Fall Gold’ in 17 weeks compared to 15 or 16 weeks of cold store-period whereas the number of lateral shoots per plant decreased. Therefore to produce more flowering lateral shoots and increase the number of flowers in ‘Autumn Bliss’ and ‘Fall Gold’, longer cold-store is beneficial. Similar findings were also reported by Takeda, (1993) that non-chilled ‘Heritage’ plants developed 15 flowering laterals while plants receiving >750 chilling units had 25 flowering laterals (Takeda, 1993). The cold storage period used in this experiment was much longer than suggested by other workers to satisfy the chilling requirements (e. g. Carew et al., 2001). Similarly cold treatment increased the main shoot growth in ‘Autumn Bliss’, ‘Erika’ and ‘Fall Gold’ to 17 weeks compared to 15 weeks and shoot height was negatively correlated with low temperature exposure (Takeda, 1993). Our results are in agreement with Carew et al., (2001) that cold storage duration increases vegetative growth rate but it was not clear whether this was because of increased duration of cold storage or because they were forced at different times in the greenhouse. Although attempts were made to maintain the temperature and light conditions in the greenhouse at similar levels for each batch during our experiment, some daily variations were observed in greenhouse conditions. In our experiment, the cold treatment was 15 to 17 weeks for 2 ±1 °C before potting under greenhouse conditions. We expected that the chilling was fully satisfied before we planted the dormant roots. The differences between the cold store periods (15, 16 and 17 weeks) could also be due to the loss of carbohydrates during cold-storage and differences in climate conditions in the greenhouse.

Photosynthetic efficiency can be linked to the growth and flowering behaviour of raspberries. For example, ‘Autumn Bliss’, ‘Fall Gold’ and ‘Polka’ had decreased F_v/F_m (efficiency of PSII) and chlorophyll a and chlorophyll a/chlorophyll b ratio which was reflected in a decreased leaf area of the leaves developed after the temperature treatment, a decreased number of unopened axillary buds per plant and a decreased main shoot height compared to reference plants.
In summary, flowering behaviour in annual-fruiting raspberries is dependent on both the chilling treatment and temperature optimum after vernalization. We hypothesised that flower induction is inhibited by high temperature stress whereas flower development is advanced in annual-fruiting raspberry cultivars which was not fully confirmed. The precise mechanisms by which high temperature affects vegetative growth and flowering is still unclear from this study due to the short period of temperature stress treatment.

12.4 Hypothesis IV: When raspberries are exposed to high-temperature for short period heat responsive genes are up-and down-regulated

When we evaluated raspberry cultivars for photosynthetic efficiency in greenhouse and climate chamber conditions, we planned to analyse the gene expression patterns of the same plant materials exposed to 27 and 37 °C for 24 h using the microarray and the real-time qRT-PCR method. The microarray was successful and a total of 40 genes significantly affected by growth at 27 and 37 °C were detected in all four annual-fruiting raspberries. These potential candidate genes belonged to different functional categories with the majority involved in plant defence and flower specific genes namely major latex like proteins (mlps) and being down-regulated at 37 °C compared to 27 °C. The mlps gene has been reported to be associated with fruit and flower development and in pathogen defence response (Lytle et al., 2009). Similarly, genes encoding electron transfer flavoprotein-ubiquinone oxidoreductase was also down-regulated which links the oxidation of fatty acids and some amino acids to oxidative phosphorylation in the mitochondria (Ishizaki et al., 2005). Down regulation of this electron transferring flavoprotein may imply that there is a reduction in electron transfer during photosynthesis at 37 °C which was confirmed by our result showing a decrease in photosynthetic efficiency ($F_v/F_m$). Volcano plotting identified 12 candidate genes that were common in ‘Autumn Bliss’ and ‘Erika’ and down-regulated at 37 °C compared to 27 °C (Figure 1, paper II). This common response in gene expression may indicate that these two cultivars can have repression of the same set of genes, probably via common signalling pathways. This could be because ‘Autumn Bliss’ and ‘Erika’ are more closely genetically related and have a common genetic background (Nikki Jennings, personal communication, Paper II).
Interestingly, two aquaporin genes (*PIP1* and *TIP2*) from the volcano list were down-regulated in ‘Autumn Bliss’ but up-regulated in ‘Autumn Treasure’, ‘Polka’ and ‘Erika’. Aquaporin facilitates the efficient transport of water molecules across membranes and facilitates passive exchange of water, compatible solute distribution and gas transfer across membranes (Johanson et al., 2001). Aquaporin contributes CO₂ conductivity of mesophyll cells (gₘ) in tobacco (Flexas et al., 2006). We did not determine the CO₂ conductance and mesophyll diffusion in our experiment. Similarly *TIPs* are a major component of the tonoplast and provide a quick equivalence of osmotic balance between cytosol and vacuolar lumen to prevent plasmolysis under hypertonic conditions (Katsuhara et al., 2008). Down-regulation of *TIPs* may be related to storage of water in vacuoles in water shortage conditions or drought tolerant cultivars. The *PIPs* are involved in the regulation of gₘ as a rapid response in drought conditions (Flexas et al., 2002). Under heat stress during day time, there is an increase in transpiration which induces a water deficit in plants and causes a decrease in leaf water potential (Tsukaguchi et al., 2003). Aharon et al. (2003) performed chlorophyll measurement in tobacco plants overexpressing an *Arabidopsis PIP1b* gene and reported a positive correlation with expression of *PIP1b* and maximum quantum efficiency of dark adapted leaves. Although we could not verify these results in raspberries, we can hypothesise a similar role for *TIP2* in raspberries. Changes in aquaporin expression may be related to the need to control water movement between storage tissues and rapidly growing and expanding tissues. In contrast, in our observation, photosynthetic efficiency was decreased in ‘Autumn Bliss’ (Paper I) and *PIP1* was up-regulated. Transcellular water flow is facilitated and regulated by aquaporins (Heinen et al., 2009). However, overexpression of aquaporins is not always beneficial to the plants. For instance, tobacco plants overexpressing *AtPIP1;2* wilt more rapidly than control plants under drought stress (Aharon et al., 2003).

An influence of aquaporin expression on photosynthetic performance of plants was also reported (Aharon et al., 2003; Flexas et al., 2006). The effects of drought and heat stress on cereals are also interlinked suggesting a common mechanism for heat, drought and other osmotic stress (Barnabas et al, 2008). Up-or down-regulation of aquaporin was reported to affect the leaf cell water permeability, water loss rate, stomatal conductance and overall leaf function (Heinen et al., 2009). Flexas et al., (2002) showed that photosynthesis and assimilation had a strong correlation with stomatal conductance (gₛ) in both field-grown and potted grapevine plants. Such a relationship may imply that down-regulation of
photosynthesis depends more on the availability of CO₂ in the chloroplast than on leaf water content or water potential.

In summary, the newly developed microarray revealed that a major response of raspberry genes to high temperatures for a short period involved down-regulation of defence related genes and up-regulation of aquaporin related genes which has not been shown before. This down-regulation of the genes seems to be a common adaptive response that enables raspberries to cope with new environmental conditions, possibly in order to conserve energy and to activate heat tolerance responses. However, a more detailed investigation of the function and regulation of specific aquaporin in leaf physiology in raspberry is still required.

12.5 Hypothesis V: Raspberry fruit yield and berry size depend on cultivar, year and production system

12.5.1 Fruit yield
In our tunnel experiments over two years, cultivars were different in their potential yield. Year and field conditions did not affect the yield in annual-fruiting cultivars but biennial cultivars were different in yield between years and field conditions. When analysing the individual cultivar, field effect in ‘Autumn Treasure’ and year effect in ‘Fall Gold’ were observed. ‘Autumn Bliss’ consistently produced the highest yield over the years and field conditions. ‘Fall Gold’ performed poorly in yield assessment across fields and years. ‘Autumn Treasure’ yielded lower compared to ‘Autumn Bliss’ which could be due to low number of berries per harvest (data not shown). In a study by Prive et al., (1993) it was reported that the yield of ‘Autumn Bliss’ increased to a maximum of 48% in proportion to their percentage of fruited laterals. The poor yield of ‘Fall Gold’ in our study could be due to low numbers of fruiting laterals (data not shown). However, the full crop potential was not realized in this experiment in ‘Fall Gold’ due to its late bearing habits. Sonsteby et al., (2009) reported that high yields were associated with cane height, the number and length of laterals, and a low proportion of dormant buds in ‘Glen Ample’. It has also been reported that the single, most important component was the length of the flowering laterals, which accounted for 82% of the yield variation. However in our tunnel experiment, we did not report the cane height and no of canes per plant.
There was a significant field x cultivar x year interaction for yield in biannual-fruited cultivars. This implies that all three factors (field, year and cultivar) contribute to the variations in yield in biannual-fruited cultivars.

12.5.2 Berry size

Berry size is one of the selection criteria in breeding for high yield in raspberries (Stephens et al., 2009) because berry size is an important yield component. It has also been reported that the larger the berry size, the higher the water content, and therefore, the lower the dry matter. In our experiments, berry size of ‘Autumn Bliss’ and ‘Fall Gold’ were different between fields, but were not different between years. On an average, ‘Autumn Treasure’ produced the largest fruit while ‘Fall Gold’ had the smallest fruit among annual-fruited cultivars.

The berry size was different between cultivar and year for biannual-fruited raspberries. ‘Octavia’ produced the largest berry size (6.0 g) in 2012 in the conventional field among four biannual-fruited cultivars. Prive et al., (1993) reported that berry count is a more important determinant of yield than berry weight.

In summary, although berry size of ‘Autumn Treasure’ was the largest, yield was lower compared to ‘Autumn Bliss’ due to a lower number of berries. The poor yield of ‘Fall Gold’ in our study could be due to low numbers of fruiting laterals. ‘Glen Fyne’ and ‘Octavia’ performed the best yield and berry size respectively among biannual cultivars. The yield and berry size depend not only on the cultivars, but also vary with production methods, for example organic or conventional fields in this present study, and with years as well.

12.6. Hypothesis VI: It is possible to produce quality organic raspberry in Danish conditions

12.6.1 Total soluble solids (TSS), titratable acid (TA) and TSS: TA ratio

The overall consumer appreciation is related more to the TSS: TA ratio than to the soluble sugar content alone. Sugar: acid ratio contributes to the characteristic flavour of a fruit and so is an indicator of commercial and organoleptic ripeness. At the beginning of the ripening process the TSS: TA ratio is low, because of low sugar and high acid content. During the fruit ripening process the fruit acids are degraded, the soluble solid and sugar content increases and the sugar: acid ratio is also increased. An increase in growth temperatures resulted in
decreased berry quality including soluble solids (TSS), titratable acids (TA), and TSS: TA ratio in strawberry fruit (Wang and Camp, 2000). It has also been reported that plants grown at 18/12 °C had the greatest amounts of fructose, glucose, and total carbohydrates in their fruit and that this decreased with increasing temperature. The greatest sucrose was found at 25/12 °C and the lowest was at 30/22 °C (Wang and Camp, 2000). In our experiments, the maximum and average temperatures were recorded to be higher by 2 - 4 °C in an organic tunnel compared to a conventional tunnel. Similarly, temperatures were higher in 2012 compared to 2011 and 2013.

12.6.2 Organic acids
The literature shows that the plant source: sink ratio, mineral fertilization, water supply, and temperature are the agro-environmental factors that have the most impact on fruit acidity (Etienne et al., 2013). Citric acid is a major organic acid of the soluble fraction in raspberries (Wang et al., 2009). Citric acid and malic acid were present at high levels when the fruit were at the immature stage and acid level decreased with increasing maturity and fruit weight (Perkins-Veazie and Nonnecke, 1992; Wang et al., 2009). With the progress of the harvest season, TSS, TA and DM increased (Remberg et al., 2010). In biannual-fruiting cultivars, citric acid was different between cultivars while malic acid was different between field conditions. Malic acid was two times higher in ‘Octavia’ in an organic field compared to a conventional field.

12.6.3 Sugars
Glucose and fructose were different between field conditions in biannual-fruiting cultivars. Most of the sugars were lower in an organic field compared to a conventional field. Sucrose content in the fruit was low and this is probably due to enzymatic hydrolysis after translocation from the leaves (Wang et al., 2009). Fructose, glucose and sucrose are the dominant sugars (5% - 6%) in raspberry fruit (Wang et al., 2009; Pritts, 2013). Metabolism of sugars and acids is closely related to the photosynthesis and respiration during ripening stages. These physiological processes have an effect on the content of sugars, acids and other nutrients in fruits (Buchanan et al., 2000). The metabolism of sugar and acids in fruit is a result of the metabolism of carbohydrate and energy. The actual amount of the metabolites is dependent on the availability and activity of the related metabolic enzymes which are influenced by environmental factors (Buchanan, 2000). Raspberry fruits grown under warm, dry summers (day temperatures near 25 °C) are sweeter, less acid, more aromatic, and more
highly coloured. It has also been reported that the effect of location on total soluble solids might be due to temperature and cultivar differences (Burrows and Moore, 2002; Bradish et al., 2012). The balance between the sugars and acids has special importance for consumer acceptability. Therefore the composition and content of organic acids and sugars are important factors influencing the organoleptic properties of fruits and juice (Lobit et al., 2006). Studies clearly indicate a higher content of total sugars in organically produced fruits such as cherries, redcurrants, and apples (Rembialkowska, 2007).

In summary, it is possible to produce quality organic raspberries with a good sugar to acid content in tunnel conditions in Denmark.
Chapter 13: Conclusions and future perspectives

13.1 Conclusion

This thesis and the papers included make it clear that growing raspberries in high temperature stress conditions reduces photosynthetic efficiency, chlorophyll concentrations and advances flowering in ‘Autumn Bliss’ and ‘Fall Gold’ while delaying it in ‘Autumn Treasure’ and ‘Erika’. A negative response to heat stress was reflected in a decreased midday F_v/F_m in all five cultivars. There was a decline in the efficacy of photosystem II under elevated temperature regimes at midday and partial recovery in the evening in ‘Polka’. Since our question of selecting heat tolerant cultivars using the F_v/F_m measurement alone could not be precisely answered, and a decrease in chlorophyll concentrations provides cross verification of the hypothesis, we would recommend both methods during selection of heat resistant raspberry cultivars. From the second part of study, it can be concluded that an elevation of temperature (∆ 10 °C) altered the expression of 40 genes (38 were down-regulated and two were up-regulated) in annual-fruiting raspberries. Two aquaporin genes (PIP1 and TIP2) were down-regulated in ‘Autumn Bliss’ but up-regulated in ‘Autumn Treasure’, ‘Polka’ and ‘Erika’. These differences will be important for future breeding of heat resistant cultivars. From the third part of the study, the field experiment, it can be concluded that ‘Autumn Bliss’ produces the highest yield consistently over the years and field conditions while ‘Octavia’ produced the largest berry size among four biannual-fruiting cultivars. ‘Fall Gold’ is the sweetest fruiting cultivar but due to low yield; its potentiality cannot be realized for commercial production. ‘Glen Fyne’ and ‘Octavia’ can be preferred for organic production as they produce high yields. ‘Autumn Bliss’ can also be recommended as it produces high yield with good fruit and taste. Therefore ‘Octavia’ and ‘Glen Fyne’ are the promising cultivars for summer and ‘Autumn Bliss’ for autumn production as protected cultivation in tunnels in Danish conditions.

13.2 Future research

This thesis project has tested the hypotheses postulated in Chapter 1 and answered the questions with the help of two sets of experiments. With the review of contemporary literature and analysis of the results from our experiment, a number of unanswered questions have been revealed that need further explanation backed up by appropriate experimental data.
Therefore in this section, some of the potential questions to be answered in future research to increase our understanding of the mechanisms of temperature stress in raspberries are discussed followed by the conclusion.

1. **Can raspberries tolerate >37 °C for longer than 7 days at field conditions followed by recovery under normal conditions?**

   Since we were not able to differentiate the raspberry cultivars in distinct categories regarding heat tolerance during our evaluation period in defined temperature levels, the stress period and temperature levels should be increased in controlled conditions (climate chamber) so that we could categorise the cultivars into two distinct groups; heat tolerant and susceptible. Moreover, our experiment should be independently repeated in field conditions to verify our conclusion from climate chamber and greenhouse conditions.

2. **Is Fv/Fm a precise physiological indicator for selection of heat resistant raspberry cultivars for breeding programs?**

   Since decreases in Fv/Fm due to abiotic factors are often complex and there are errors associated with measurement, the Fv/Fm should be evaluated coupled with other non-invasive tools such as absorption spectroscopy, gas exchange analyses, and infrared thermography. Moreover, in our research, we used dark adapted leaves for Fv/Fm measurements, and therefore, light adapted values should also be considered for better understanding of the heat stress mechanism in raspberries.

3. **Is there any effect of seven days heat stress during flower induction into fruit yield and berry quality?**

   Even though, in our research, some cultivars showed early opening of terminal flowers and some cultivars showed delayed opening compared to reference plants in greenhouse conditions, our project failed to explain the extension of the flowering period because of our heat stress treatment for a seven day period. So our experiment should be independently repeated to assess the extension or shortening of flowering and fruiting period within a season, berry yield and quality parameters such as total soluble solids, titratable acid, sugar, and organic acid, and polyphenol composition.
4 Can we validate number of heat responsive genes observed in microarray analysis by using real-time qRT-PCR?

We attempted to validate two major heat responsive genes, PMP and TIP2 that were expressed in microarray analysis by using qRT-PCR. This method is needed to verify that the expression values observed from the microarray data actually represent a set of differentially regulated genes. The PCR primer and probe design for qRT-PCR was difficult due to the fact that there was unanticipated allelic variation in the genes selected for observation. Therefore, it is necessary to repeat the qRT-PCR to improve the number of genes observed and validated.

New primer and/or probe sets are necessary using conserved regions, flanking or omitting the variation between distantly related genotypes. This may require empirical testing, or sequencing each gene in each accession, to ensure accurately designed qRT-PCR probes.

5 How can the yield and quality of organic production be maintained? Will the yield decrease due to diseases? How long will we be able to obtain high yields in raspberries from an organic field?

One of the major bottlenecks in organic raspberry production in tunnel conditions is the occurrence of many pests, and the lack of economically viable pest control methods. Many pesticides made from plant extracts, like azadirachtin and pyrethrum, are available in organic growing, but are not being allowed for use under Danish conditions. Therefore, growers starting an organic raspberry production must do so without any documented control measures for pests like the raspberry beetle (Byturus tomentosus), red spider mites, and aphids (Aphis idaeus and Amphorophora idaei, direct pests as well as virus vectors). There is little knowledge on how to use them in a way that works in Danish conditions. The challenge of disease and pests is supposed to be increased due to increased temperature in tunnel conditions and therefore it needs to introduce disease and pest resistant cultivars for commercial production. Therefore, it is necessary to evaluate the organic production methods and the use of alternative methods to control pests to increase our understanding of the possibility of sustainable organic raspberry production in Denmark.

In our experiment II, organic yield and quality were compared and evaluated only for two consecutive seasons. A valid conclusion for yield and quality can only be precisely
established if we extend the production period for more years as diseases and pests could increase in an organic field compared to a conventional field.

6 Future research should also be aimed at analysing the polyphenol content of the commercial raspberries produced in organic and conventional tunnels in Danish conditions.
References


Supplementary Figure. 1

Conventional PCR amplification of a single amplicon from the total cDNA samples of ‘Autumn Bliss’ and ‘Erika’ for aquaporin (*TIP2*), *GAPDH* and a plasma membrane protein. Duplicate samples of a single product from each total cDNA are shown. ‘Lane: 1, DNA markers; lanes 2, 3, aquaporin gene (*TIP2*) at 27°C; lanes 4, 5, aquaporin gene (*TIP2*) at 37°C; lanes 6, 7, *GAPDH* (reference gene) at 27°C; lanes 8, 9, *GAPDH* (reference gene) at 37°C; lanes 10, 11, plasma membrane protein gene at 27°C; and lanes 12,13, plasma membrane protein gene at 37°C.
Supplementary File 1

Differentially expressed and n-fold changes in gene expression profiles of four annual-fruiting raspberry cultivars after 24 h of heat stress at 27 and 37°C

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<td>JHI_Ri_ASM02Jun2011_MMB_26875</td>
<td>TIP2;1, DELTA-TIP1, AQP1, ATTIP2;1, DELTA-TIPDELTA-TIP (delta tonoplast integral protein); water channel</td>
<td>aquaporin TIP2 [Malus prunifolia]</td>
<td>Water and solute movement</td>
<td>6.4E-44</td>
<td>-3.2 1.3 1.1 1.4</td>
</tr>
<tr>
<td>40</td>
<td>JHI_Ri_ASM02Jun2011_MMB_31516</td>
<td>ATPIP1, PIP1, PIP1;1, PIP1APIP1A (PLASMA MEMBRANE INTRINSIC PROTEIN 1A)</td>
<td>aquaporin [Fragaria x ananassa]</td>
<td></td>
<td>0.00738</td>
<td>-2.4 1.4 1.2 1.2</td>
</tr>
</tbody>
</table>

†A fold-change value is the ratio of the normalized value at 37°C divided by the corresponding value at 27°C; a fold-change value that is less than one was replaced by the negative of its inverse. Positive values denote gene up-regulation and negative values denote gene down-regulation.