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Project leaders:	Dr Rob Hancock Scottish Crop Research Institute Invergowrie Dundee DD2 5DDA Dr Chris Atkinson East Malling Research New Road East Malling, Kent ME19 6BJ
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Key workers:	P.G. Walker, S.D.A. Pont, N. Marquis (SCRI) M.J. Davies, H. Longbottom, J.M. Taylor, Y.Y. Ford, N.A. Hipps, P.A.A. Dodds (EMR)
Project location:	SCRI - Dundee, Angus EMR - East Malling, Kent
Project co-ordinator:	Michael Dunsire GlaxoSmithKline GSK House Brentford, Middlesex TW8 9GS
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Collaborators:	GlaxoSmithKline Nutritional Healthcare Scottish Crop Research Institute East Malling Research Mylnefield Research Services Horticultural Development Council representing the Blackcurrant Growers Association
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The following are members of the Consortium for LINK project MRS/003/02

1. GlaxoSmithKline Nutritional Healthcare
2. Scottish Crop Research Institute
3. East Malling Research
4. Mylnefield Research Services Ltd
5. Horticulture Development Council on behalf of the Blackcurrant Growers Association

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CONTENTS

GROWER SUMMARY

MRS/003/02

Development of physiological and agronomical tools for increasing the L-ascorbic acid yield from blackcurrant bushes

FINAL REPORT 2007

GROWER SUMMARY

HEADLINE

- Vitamin C accumulates in blackcurrant fruit by *in situ* synthesis from imported sugars during the early stages of fruit development (green fruit)
- Fruit vitamin C concentration is tightly regulated and under strong genetic control
- Yield is the overriding factor controlling total vitamin C production per bush
- Excess bush nitrogen had a negative impact on fruit vitamin C and total bush vitamin C yield
- Irrigation increased fruit yield
- Irrigation does not increase berry vitamin C concentration, but does enhance total vitamin C yield per bush
- Genetic markers have been developed to allow more rapid selection of high vitamin C cultivars

BACKGROUND AND EXPECTED DELIVERABLES

The fortification of food and drinks will soon be regulated by EU directive (COM(2003) 671 final). This legislation will provide an opportunity for expansion of the blackcurrant juice market due to its high natural vitamin C content. In addition, there is a desire on the part of processors to obtain a higher vitamin C product. However, the vitamin C concentration of blackcurrant juice varies enormously between varieties, seasons and growth locations. The objectives of the project were therefore to obtain a sound fundamental understanding of the factors that control the accumulation of vitamin C in blackcurrant fruit in order to inform experiments to provide:

1. An agronomic blueprint for the maximisation of blackcurrant fruit vitamin C content
2. A firm basis for the breeding of blackcurrant germplasm with enhanced vitamin C content

SUMMARY OF THE PROJECT AND MAIN CONCLUSIONS

The project was divided between two research centres, the Scottish Crop Research Institute (SCRI), Dundee, which has expertise in plant physiology, plant biochemistry and blackcurrant breeding and East Malling Research (EMR), Kent, which has expertise in woody perennial physiology, top and soft fruit breeding and horticultural research. The structure of the project was such that research into the physiology and biochemistry of vitamin C accumulation in fruit undertaken at SCRI would provide information to aid the design of agronomic experiments undertaken at EMR to provide growers with crop management techniques for the optimisation of blackcurrant fruit vitamin C. In the longer term, data generated by the project was also designed to aid the ongoing blackcurrant breeding programs at both sites.

Work at SCRI focused on determining the mechanism of vitamin C accumulation in blackcurrant fruit and the reason that some cultivars have a higher fruit vitamin C concentration than others. Two major mechanisms for fruit vitamin C accumulation can be considered; these are net synthesis within the fruit from imported sugars or alternatively direct import of vitamin C to the fruit from external sources (stored vitamin C, leaves). By understanding how and when vitamin C accumulation occurred in blackcurrant fruit it was intended to guide agronomic practice and by understanding why some cultivars have higher fruit vitamin C concentrations than others it was intended to provide tools to accelerate the development of new high vitamin C cultivars.

The research at East Malling Research (EMR) focused on developing an understanding of agronomic and practical ways to enhance fruit vitamin C and how the results of this work could be turned into commercially viable growing systems. A major aim of the work was to utilize the knowledge obtained from the biochemical studies undertaken at SCRI and to integrate this science into practical novel growing systems. Four major field experiments were established at East Malling to determine the effects of manipulating nitrogen nutrition, crop load, irrigation and the use of light reflective mulches respectively.

The mechanism of vitamin C accumulation in blackcurrant fruit

A number of possibilities were considered for the mechanism vitamin C accumulation in blackcurrant fruit:

1. The mobilisation of stored vitamin C in roots and stems and transfer to fruit
2. The transport of vitamin C synthesized in leaves to fruit
3. The synthesis of vitamin C from transported sugars within the fruit

Hypothesis 1, the mobilization of stored fruit was rejected because detailed examination of the tissue distribution of blackcurrant plants throughout the growing season revealed that insufficient vitamin C was present in the plant during the winter to account for the vitamin C accumulated in fruit during spring.

Hypothesis 2, the transport of vitamin C from leaves to fruit was investigated in detail. Although work using radioactive tracers demonstrated that the vitamin could move from leaves to fruit other work suggested that the amount of the vitamin transported did not significantly contribute to the final fruit vitamin C content.

- No differences were observed in the amount of vitamin C transported between blackcurrant cultivars containing low and high concentrations of fruit vitamin C
- During fruit vitamin C accumulation, export from leaves was negligible
- At the time of maximum vitamin C accumulation in fruit, its biosynthetic capacity in leaves decreases

Hypothesis 3, the synthesis of vitamin C within the fruit was examined by the use of radiochemical tracers. Fig. G1 clearly shows that there was decline in fruit biosynthetic capacity associated with cessation of vitamin C accumulation. Furthermore, there was a correlation between final fruit vitamin C accumulation and biosynthetic capacity in high and low vitamin C cultivars.

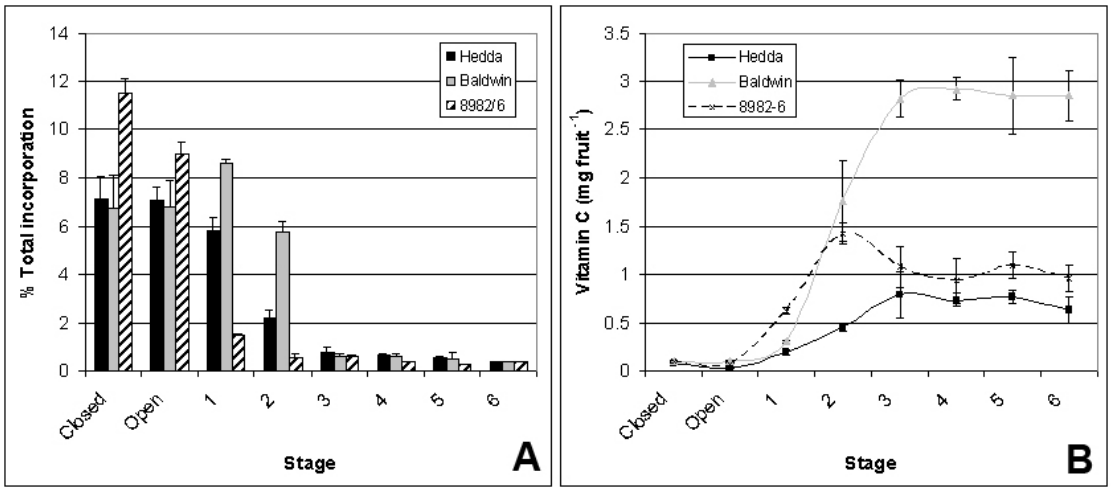


Figure G1. The relationship between vitamin C biosynthesis and vitamin C accumulation in blackcurrant fruit

Panel A shows the vitamin C biosynthetic capacity of 3 different genotypes of blackcurrant fruit at different stages in development as determined by the quantity of radioactive mannose (a vitamin C precursor) incorporated into vitamin C after a 4 h incubation. Panel B shows vitamin C accumulation in fruit of the same three genotypes. Closed = closed flowers, open = open flowers, 1 = small green fruit, 2 = large green fruit, 3 = green-red fruit, 4 = red-green fruit, 5 = red fruit, 6 = black (ripe) fruit.

Control of fruit vitamin C accumulation

Several lines of evidence suggest tight genetic control of fruit vitamin C accumulation. Firstly, an analysis of historical records showed that cultivar hierarchy was generally maintained year on year with regards to fruit vitamin C concentration. Secondly, manipulation of sugar availability by post-harvest defoliation resulted in the production of fruit with identical vitamin C levels in the following year but only approximately half the yield. These data strongly suggest that the plant will maintain fruit vitamin C concentration at the expense of fruit yield. Finally, when fruit vitamin C concentrations were artificially raised by supplying a vitamin C precursor the levels rapidly returned to those found in untreated plants following removal of the precursor. This suggests the fruit has an ideal vitamin C level and the plant is able to alter fruit metabolism in order to maintain that level. These findings have several important implications:

i. Agronomic manipulation of fruit vitamin C levels are likely to prove difficult.

ii. As fruit vitamin C concentration is under tight genetic control, breeding for high fruit vitamin C is a feasible objective.

Impact of agronomic manipulation on fruit vitamin C concentration

i. Manipulating nitrogen supply

Using nitrogen at 'excessive' levels promoted bush growth, particularly leaf canopy development. Fruit berry size also increased with bush nitrogen status, but at the expense of fruit vitamin C concentration which declined with increasing nitrogen. Field-based experiments showed that at a supply rate greater than 152 kg N ha^{-1} there was no value (particularly yield gain) of adding more nitrogen.

ii. Manipulation of crop load

Crop load adjustment (fruit removal) showed that a 'compensatory capacity' of increasing berry size to offset the initial potential reduction in yield occurred, but varied with cultivar. A reduction in strig fruit number did not change the berry vitamin C concentration. This was also the case when natural variation in strig berry number (due to 'run-off') was examined. Experiments show that most of the carbohydrate for fruit growth came from leaves at the tip of the shoot, while between 20 to 30% came from the strig leaves with the remainder from 'storage' (woody tissues).

iii. Manipulation of water supply

A range of different irrigation regimes were set up in the field to achieve differences in soil moisture content and these impacted on vegetative shoot growth. Bushes not receiving full irrigation had reduced shoot growth. Partial drought did not influence fruit yield per bush. However, over time optimal irrigation had cumulative positive effects on fruit yield. There was no impact on berry vitamin C concentration, but a large positive enhancement on total vitamin C yield per bush.

iv. Manipulation of light interception

Bush light interception was increased by the use of light reflective plastic soil mulches. These materials were effective in reflecting light back into the leaf canopy. Mulches increased bush canopy growth and were accompanied by a relatively small increase in fruit yield. There was no influence on fruit vitamin C concentration, or total bush yield of vitamin C initially but over time (two seasons) total bush vitamin C yield increased.

v. Developing strategies for maximisation of vitamin C production in the field

A number of field-based experiments were carried out throughout this project to evaluate commercially practical approaches to enhance fruit vitamin C concentration. Field-based approaches were also used to manipulate the nutritional constituents of plant tissues and fruits. None of these 'nutritional supplement' approaches (phosphorus, potassium or calcium) have revealed any significant positive influences on yield or fruit vitamin C concentration. Irrigation and light reflective mulches were chosen as the most appropriate and practical combination option for a field planting designed to enhance fruit yields and the total yield of vitamin C. This combination of treatments produced significant enhancements to fruit yield and the total yield of vitamin C per bush.

Genetic markers for accelerated blackcurrant breeding

A detailed analysis of gene sequences for several genes required for vitamin C biosynthesis in high and low vitamin C genotypes revealed a specific sequence associated only with high vitamin C genotypes. Analysis of the gene in seedlings of novel crosses will allow determination of the fruit vitamin C status at a much earlier stage in plant development (seedling stage). The result will be that within breeding programs more crosses can be performed each year and those seedlings that do not contain the appropriate sequence can be discarded. The impact will be the more rapid development of novel blackcurrant cultivars containing acceptable levels of vitamin C in conjunction with other desirable traits.

Conclusions

- Blackcurrant fruit vitamin C is accumulated during the fruit expansion stage

- There is a correlation between fruit biosynthetic capacity and fruit vitamin C content at harvest between different genotypes
- Fruit ascorbic acid concentration is closely regulated and under genetic control
- Agronomically induced enhancements of fruit ascorbic acid concentration were small irrespective of treatment and cultivar
- Despite being able to manipulate fruit size (yield) by altering the source of carbohydrate (leaf removal etc) for growth there was no effect of these treatments on vitamin C concentration
- The apparent control of fruit vitamin C concentration suggests that if yields of vitamin C are going to be increased agronomically then this is most likely to be successful through an increase in fruit number not concentration of vitamin C
- Predictive markers have been developed for the accelerated breeding of high vitamin C cultivars

FINANCIAL BENEFITS

Changes to agronomic practice can enhance yield. The financial benefits of such changes are dependent on the costs of implementing the changes versus the additional generated from enhanced yields.

ACTION POINTS FOR GROWERS

- Monitor bush and soil nitrogen status
- Determine the economic returns from the use of reflective mulches
- Irrigation options should be considered, particularly with respect to drought and climate change
- Monitor the availability of new cultivars

MILESTONES

Task	Objectives	Duration	Proposed Date	Actual Date
1.3	Complete preliminary distribution and transport studies		31.10.03	31.10.03
1.7	Complete distribution and transport studies		31.10.04	31.08.05
1.21	Complete characterisation of annual starch storage cycle in two blackcurrant cultivars		31.03.07	31.03.07
1.22	Determine leaf export capacity during AsA accumulation in blackcurrant fruit		31.10.06	31.10.06
2.5	Identify correlative trait of berry AsA		31.10.04	31.10.04
2.8	Finish screening mutagenised populations		11.02.05	11.02.05
2.9	Identification of correlative traits		31.10.05	31.10.05
2.11	Identification of AsA markers		31.06.07	31.09.07
2.11a	Quantify AsA, organic acids and glutathione in leaf tissues of a 200 seedling population grown under a controlled environment.		31.10.06	31.10.06
2.11b	Determine the antioxidant capacity of a 200 seedling population grown under a controlled environment		31.10.06	31.10.06
2.11c	Determine the metabolite profile of a 200 seedling population grown under a controlled environment		31.03.07	31.09.07
2.11d	Determine critical enzyme activities in AsA synthesis and turnover in leaf tissues of a 200 seedling population grown under a controlled environment.		31.03.07	31.03.07
2.11e	Determine gene expression of four critical biosynthetic genes during fruit development in three blackcurrant genotypes		31.09.06	31.09.06
2.11f	Identify polymorphisms in blackcurrant AsA biosynthetic genes associated with low or high fruit AsA		31.05.07	31.09.07
3.4	Complete pilot experiment		31.03.03	31.03.03
3.7	Suitable potential agronomic manipulation techniques identified		31.03.04	31.03.04
3.9	Complete temperature studies		31.05.07	31.05.07
4.3	Complete data processing regional survey		31.03.04	28.02.05
4.5	Complete analysis field trial		31.03.05	31.03.05
4.7	Complete analysis of all field trials		03.04.06	03.04.06
5.3	Complete grower field trial (EMR)		07.12.06	07.12.06
5.4	Develop mechanistic understanding		31.06.07	31.01.06
5.5	Deliver final report		30.09.07	15.10.07

Describe the overall objectives which have changed and give the reasons for the changes:

Several objectives changed during the course of the project as described in the document Realignment of Horticulture Link MRS 002/03 (Appendix 1). These changes were raised

at the management committee meeting and approved following full discussion. Revised proposals were submitted to all funders and consortium members and approved in full.

SCIENCE SECTION

INTRODUCTION

Commercial objectives

The vast majority (>95%) of the UK blackcurrant crop is used for processing to blackcurrant juice drinks. Part of the marketing strategy for such products is the high vitamin C (L-ascorbic acid; AsA) content of blackcurrants and blackcurrant juice. EU directive COM(2003) 671 final is designed to regulate the fortification of foods and drinks throughout the EU and may well impact on the formulation of blackcurrant juice drinks. Therefore, there is a drive from blackcurrant processors for maximisation of the vitamin C (L-ascorbic acid; AsA) content of blackcurrant fruit. The current project seeks to address these needs by developing a thorough knowledge of the biochemical and physiological factors resulting in AsA accumulation in blackcurrant fruit. In the short term, this knowledge will aid the design of agronomic experiments aimed at the development of crop management techniques resulting in maximisation of fruit AsA content. In the longer term, detailed knowledge of the process resulting in fruit AsA accumulation will aid in the development of physiological, biochemical and molecular markers in order to inform and accelerate breeding programs for fruit that combines high AsA with other desirable traits.

Scientific background

Although knowledge regarding the biochemistry of AsA synthesis and recycling has made tremendous advances in recent years, much of the work has been undertaken in photosynthetic tissues using model plants such as *Arabidopsis thaliana* and very little attention has been devoted to understanding the mechanisms governing the accumulation of AsA in sink tissues such as fruits and tubers. Similarly, although a number of studies have examined the effect of agricultural practice on the AsA content of a number of crops (Lee & Kader, 2000), this area has not previously been the subject of systematic study. Below is a brief outline of the current state of knowledge regarding biochemical and physiological mechanisms of AsA accumulation in plants and of agronomic factors previously demonstrated to influence the AsA content of crops.

In principle, accumulation of AsA in blackcurrant fruit could be influenced by three major factors namely; (i) *in situ* biosynthesis from imported sugars, (ii) transport of AsA from photosynthetic tissues or storage pools via the phloem and (iii) AsA turnover and recycling within the fruit.

The primary biosynthetic pathway for AsA in plants was discovered in 1998 (Wheeler *et al.*, 1998) and since then a number of alternative pathways have been presented, which may be specific to particular organs, tissues or developmental stages (Agius *et al.*, 2003; Wolucka & van Montagu, 2003; Lorence *et al.*, 2004) (Fig. 1). At the present time, no precise data regarding the contribution of alternative pathways to the AsA pool is available, although data from *A. thaliana* mutants suggest that their contribution is limited (Conklin *et al.*, 1999). Furthermore, little is known regarding the control of AsA biosynthesis although a number of reports have described effectors of several biosynthetic enzymes (Wolucka & van Montagu, 2003; Mieda *et al.*, 2004) and evidence has been presented suggesting the metabolic integration of respiration, mitochondrial electron transport and AsA biosynthesis mediated via the ultimate biosynthetic enzyme, L-galactono-1,4-lactone dehydrogenase (Millar *et al.*, 2003).

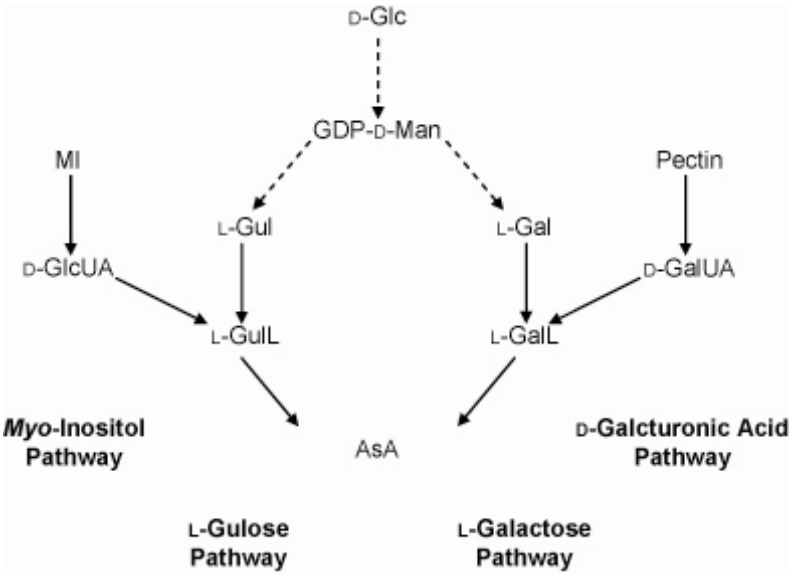


Figure 1 Schematic of AsA biosynthetic pathways in plants

Phloem transport of AsA was first demonstrated using radioactive tracer in *A. thaliana* and *Medicago sativa* (Franceschi & Tarlyn, 2002) and further detailed in potato where it was shown that the phloem AsA concentration exhibited a diurnal fluctuation similar to that observed in leaves (Tedone *et al.*, 2004). Similarly, artificial enhancement of leaf AsA content in potato resulted in increased phloem and developing tuber AsA concentrations suggesting that AsA transport could be a mechanism of accumulation in sink tissues such as tubers and fruits. A novel additional mechanism by which AsA could accumulate in phloem was the demonstration that this tissue was highly active for AsA synthesis (Hancock *et al.*, 2003).

AsA catabolism occurs when the unstable oxidised form, dehydroascorbic acid (DHA) is hydrolysed to form 2,3-diketogulonic acid. This reaction is known to occur spontaneously, however plants also use AsA as an intermediate in the synthesis of organic acids such as oxalate and tartrate (Hancock & Viola, 2005) and recent work has demonstrated that these metabolic pathways are enzymatically catalysed (Green and Fry, 2005). As an alternative to catabolism, DHA can be recycled to AsA through reduction by the tripeptide glutathione catalysed by dehydroascorbate reductase (DHAR). That such recycling contributes to the steady-state AsA pool was demonstrated by the overexpression of wheat DHAR in either tobacco or maize which resulted in up to a four fold increase in foliage AsA content (Chen *et al.*, 2003).

Of the environmental parameters studied, light has been shown to have an influence on the AsA concentration of apple tissues where exocarp AsA concentration was dependent on where on the apple circumference it was sampled with those parts receiving most sunlight by virtue of their position containing approximately twice as much AsA as those parts receiving least sunlight (Davey *et al.*, 2004). Increasing the amount of available light in strawberry using reflective mulches has been demonstrated to increase the fruit AsA concentration in some cultivars but not others (Atkinson *et al.*, 2006). Similarly, light intensity has been shown to affect the AsA concentration of leaves (Smirnoff & Pallanca, 1996) which through AsA transport may also affect fruit AsA concentration as was shown for potato tubers (Tedone *et al.*, 2004). The effects of temperature on fruit AsA levels have not been widely studied however temperature elevation in controlled environment chambers resulted in reduced AsA content in blackcurrant (Redalen, 1993) and kiwifruit

(Richardson *et al.*, 2004). Mild water stress generally appears to increase the AsA content of crops (e.g. Sorensen *et al.*, 1995) although we are unaware of any studies undertaken on fruit. Enhanced nitrogen nutrition has been shown to positively correlate with the levels of AsA and other antioxidants in strawberry fruit (Wang & Lin, 2003) although the opposite effect was observed in citrus fruits and tomato (Nagy, 1980; Toor *et al.*, 2006). Finally, elevated CO_2 was demonstrated to enhance the AsA content of strawberries (Wang *et al.*, 2003).

Very little is known regarding the genetics of AsA accumulation in fruit tissues although recently, several quantitative trait loci (QTL) were identified in apple. Three QTLs were found in both Telamon and Braeburn cultivars that accounted for up to 60% of the total observed variance in fruit AsA concentration. One of the QTLs identified was linked to a locus governing general plant and fruit growth while the others did not coincide with other known fruit quality traits (Davey *et al.*, 2006).

Given the lack of knowledge regarding the biochemical and physiological parameters involved in AsA accumulation and distribution at the whole plant level, the primary aim of the project is to address these questions in blackcurrant with particular reference to accumulation of AsA in fruit. This knowledge will be used to guide experiments to determine optimal agronomic practice for maximising fruit AsA concentration and to provide underpinning knowledge for the generation of new high AsA blackcurrant cultivars.

Detailed project objectives

1. Determine how, where and when AsA is made in the plant

Biochemical and physiological analyses will be performed to determine sites and stages of ascorbic acid (AsA) accumulation during plant development. Radiolabelled precursors of AsA biosynthesis and metabolic profiling of phloem exudates will be used in experiments to investigate AsA biosynthesis and turnover in individual tissues, and whether short- or long-distance transport is involved in accumulation of AsA in the berries.

2. *Screen available germplasm and identify early correlative and predictive markers of AsA berry content*

Populations of *Ribes nigrum* segregating for fruit AsA content, and a number of mutagenised *Ribes* selfed lines developed in another related project will be screened for AsA content in berries and other tissues to identify correlative traits and potential for pre-fruiting identification of high AsA genotypes.

3. *Define internal and external factors affecting berry AsA content*

Physiological experiments on potted plants will be performed to alter dry matter distribution and to determine the effect of this on berry AsA content. This will be achieved via manipulating (i) soil nitrogen availability, (ii) air temperature, (iii) soil water availability, and (iv) crop load. The experiments span years 1-4 and will be refined, using data from pilot experiments and from Objective 1. These experiments will provide whole plant information on the control and development of AsA production at the bush level, and will be used to determine the approach in Objective 4.

4. *Identify agronomic practices for optimisation of AsA crop yield*

Agronomic manipulations will be tested for their potential to enhance berry AsA content, via field-based experiments. The methods used will be based on the results from Objectives 1 and 3, and reflective mulches to increase light intensity at the bush level will also be used. An analysis of historical data from a regional study of crop yield, berry juice and AsA content will be performed.

5. *Develop strategies for maximisation of AsA production*

The insights gained from Objectives 1-4 will be used to develop a crop management system to enhance AsA production in blackcurrant fruit. This will be tested on grower sites.

MATERIALS AND METHODS

Plant material

Ribes nigrum cv. Baldwin and genotype 8982-6 were obtained from Welsh Fruit Stocks, Hereford, UK and cv. Hedda was obtained from Planteforsk, Kiseveien, Norway. Plants were declared free of reversion virus and planted out in the field at SCRI in October 2002. Standard industry pest control, fertilisation and irrigation conditions were applied. A number of plants were potted into compost and used for laboratory work. Field experiments at EMR involved the cvs Ben Lomond (3-year-old) and Baldwin (4-year-old).

In order to determine predictive biochemical markers for fruit AsA concentration an experimental population consisting of 400 individuals from a Ben Starav × Ben Gairn cross were germinated in winter 2005. The plants were potted out, grown on under glass and hardened by standing out in September 2006. In January 2007, the population was transferred to the field at SCRI, Dundee.

Plant sampling for sugar and starch determination

Three plants of cv. Baldwin were sampled at various points throughout the annual cycle. Plants were removed from the field and brought to the laboratory where excess soil was removed under running water. Plants were dissected into their component parts (buds, leaves, flowers, fruit, old-growth stems, new growth stems and roots), tissues were cut into appropriate sizes and immediately frozen in liquid N_2. Tissues were then lyophilised and stored at -80^{o}C until extraction.

In a further experiment, three field grown plants of cv. Baldwin were defoliated immediately after harvest in August 2005. Plants were periodically examined and any renewed leaf growth immediately removed until natural leaf drop at the end of October 2005. Plants were then left in the field and allowed to develop as normal throughout the growing season in 2006. At ripeness, fruit were harvested and water content determined by weight before and after lyophilisation. Sugar, starch and AsA concentrations were determined as outlined below.

Extraction and quantification of sugars and starch

Woody tissues were milled and soft tissues were ground to a powder in a mortar and pestle. Tissues were then extracted in 80% ethanol (19:1 v/w) at 80^{o}C for 1 h with occasional shaking. Samples were centrifuged (10000 g, 20 min, 1^{o}C) and the supernatant reserved. Pellets were re-extracted (20:1 v/w) and both supernatant fractions combined. Ethanol was removed at 50^{o}C under reduced pressure and the aqueous fraction frozen and lyophilised. Samples were resuspended in 10 volumes of distilled H_2O and the glucose, fructose and sucrose concentration estimated enzymatically as previously described (Viola & Davies, 1992). Tissue pellets were washed in distilled H_2O, resuspended in nine volumes of distilled H_2O and starch was gelatinised by boiling for 2 h. The samples were cooled and 1 volume of 1 M acetate buffer pH 5.0 was added. Amyloglucosidase (Megazyme, Co. Wicklow, Ireland) was added to a final concentration of 10 U ml^{-1} and incubation continued for 18 h at 37^{o}C. Pellets were removed by centrifugation and glucose released from starch in the supernatant was analysed enzymatically (Viola & Davies, 1992).

Extraction and quantification of AsA and organic acids

Analyses at SCRI were undertaken as follows. Tissues were ground to a fine powder in liquid N_2 and extracted in 5% (w/v) metaphosphoric acid (MPA) containing 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (19:1 v/w). Samples were centrifuged (16000 g, 5 min, 1^{o}C) and AsA and other organic acids in the supernatant quantified by HPLC according to Hancock *et al.* (2003).

For the large-scale analysis of AsA as undertaken at EMR, approximately 20 g frozen weight of berries were homogenised in a blender with 10 volumes of 25 mM ammonium acetate buffer pH 4.0. The homogenate was centrifuged at 12000 rpm in a cooled (4^{o}C) ultracentrifuge for 15 minutes, and the volume of the resulting supernatant was measured. A 3 ml aliquot was taken from the supernatant. 300 µl of a 5% trifluoroacetic acid (TFA) aqueous solution and 30 µl of a 500 mM solution of the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were added to the 3 ml aliquot, which was left at 4^{o}C for 30 min. 500 µl of the resulting solution was taken by pipette and placed in a

Whatman Mini-UniPrep HPLC vial incorporating a 0.45 μm polypropylene filter. HPLC conditions were as follows: Varian Chromsep Polaris C18-A 3μ 150 × 2 mm column (C18 column with a polar embedded phase) fitted with a Chromsep 10 × 2.0 mm Polaris C18-A 3μ guard cartridge; column temperature 30^{o}C; mobile phase 0.5% TFA in aqueous solution run isocratically with a flow rate 0.2 ml min^{-1}. The chromatographic system consisted of a Waters Alliance 2690 HPLC system connected to a Waters 996 photodiode array detector. Detection of AsA was at 245 nm, and quantitative analysis was by means of an external standard curve.

Quantification of glutathione

Glutathione was quantified in MPA extracts from leaf discs by HPLC combined with coulimetric detection as described by Potesil *et al.* (2005) with a number of modifications. Filtered samples (10 μl) were injected onto a Luna C18 3μ 150 x 2.0 mm HPLC column (Phenomenex, UK) and eluted for 10 min at 0.18 ml min^{-1} in 0.05% trifluoracetic acid (TFA) followed by a step gradient to 97% 0.05% TFA and 3% methanol for 10 min after which the column was re-equilibrated in 0.05% TFA for 10 min. Coulimetric quantification was achieved using an ESA 5600A CoulArray detector (ESA Analytical ltd., UK) at an electrode potential of 600 mV. Its identity was confirmed in leaf extracts by co-elution with authentic standards and by the similarity of signal ratios from electrodes set at 400, 500 and 600 mV with those of an authentic standard.

Extraction and quantification of total antioxidants

Leaf discs were lyophilised and the dried tissue ground to a powder using a micropestle in a 1.5 ml tube. 0.5 ml distilled H_2O was added and extraction continued at 80^{o}C for 2.5 h with vigorous shaking by vortex every 30 min. Samples were cooled, centrifuged (10000 g, 5 min, 4^{o}C) and the supernatant was removed (aqueous fraction). The pellet was resuspended in 0.5 ml 50% methanol and extraction repeated as previously described. Samples were collected and centrifuged, the supernatant was retained (lipophillic fraction) and the extraction repeated in 50% methanol containing 2.5 M HCl (wall-bound fraction). Total antioxidant capacity in each fraction was determined by measuring absorbance (750 nm) following reaction of 0.25 ml of appropriately diluted samples with an equal volume

of Folin-Ciocalteu reagent (Sigma-Aldrich, UK). Following standing for 3 minutes, 2 volumes of saturated Na_2CO_3 were added and colour was allowed to develop for 1 h in the dark prior to absorbance measurements. Total antioxidant capacity was determined by reference to a standard curve constructed using Trolox.

Metabolite profiling

Leaf discs were ground to a fine powder in liquid N_2 and grinding continued in 2 ml of 60% methanol containing 0.5% formic acid and 10 μM reserpine, 200 μM fluoro-tyrosine, 200μM fluoro-tryptophan and 500 μM morin as internal standards. Samples were transferred to a blood rotator and gently shaken at 4°C for 30 min. Samples were centrifuged (16000 g, 5 min, 1°C) and the supernatant transferred to a fresh tube. Methanol was removed under reduced pressure at room temperature and the samples were then lyophilised and stored at -20°C prior to further analysis.

Samples were resuspended in 250 μl 5% acetonitrile and 20 μl were injected onto a Synergi 4 μ HydroRP 150 x 4.6 mm reverse phase column. Mobile phases were 0.05% tri-fluoracetic acid (TFA) in water (buffer A) and 0.05% TFA in methanol (buffer B). A gradient was run at a constant flow rate of 0.4 mL min^{-1} as follows; 0 min 5% B, 60 min 70% B, 61 min 100% B, 69 min 100% B, 71 min 5% B, 75 min 5% B. Metabolites were detected by PDA in the region 200-600 nm and by negative ion mode mass spectrometry in the range 80-2000 kDa.

Enzyme extraction and assay

Tissues were ground to a powder in liquid N_2 and extracted in three volumes of 100 mM tris pH 7.5, 100mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1mM EGTA, 1% PVPP and protease inhibitor tablets (Roche, UK). Samples were centrifuged (10000 g, 10 min, 1°C) and either used directly for enzyme analysis or alternatively desalted on PD10 gel filtration columns (Roche, UK) prior to analysis.

L-Galactose dehydrogenase activity was determined by measurement of the change in optical density at 340 nm in a solution consisting of 50 mM HEPES buffer pH 8.0

containing 5 mM $MgCl_2$, 0.5 mM NAD and an appropriate amount of enzyme extract. The reaction was started by the addition of L-galactose to a concentration of 2 mM.

Monodehydroascorbate reductase activity was determined by measurement of the change in optical density at 340 nm in a solution consisting of 50 mM potassium phosphate buffer pH 7.5 containing 2 mM NADPH, 1 mM AsA and an appropriate amount of enzyme extract. The reaction was started by the addition of 0.2 U ml^{-1} ascorbate oxidase (Roche, UK).

Dehydroascorbate reductase activity was determined by measurement of the change in optical density at 265 nm in a solution consisting of 50 mM potassium phosphate buffer pH 7.5, 0.5 mM DHA and an appropriate amount of enzyme extract. The reaction was started by the addition of reduced glutathione to a concentration of 1 mM.

Glutathione reductase activity was determined by measurement of the change in optical density at 340 nm in a solution consisting of 50 mM potassium phosphate buffer pH 7.5 containing 0.2 mM NADPH, 1 mM EDTA and an appropriate amount of enzyme extract. The reaction was started by the addition of oxidised glutathione to a concentration of 5 mM.

Leaf labelling and autoradiography

Leaves were excised from field-grown plants during the green fruit expansion phase. The upper leaf surface was lightly abraded with carbarundum powder and discs were cut using a 20 mm diameter cork borer. Leaf discs were floated upper surface down on a buffer solution containing 25 mM MES pH 5.5, 20 mM $CaCl_2$ and incubated under light for 15 h as described by Hancock *et al.* (2003). Following light incubation, 1 mM *p*-chloromercuribenzenesulphonate (PCMBS) was added to appropriate treatments and incubation was continued for 30 min prior to the addition of 1 mM U-[^{14}C]sucrose (specific activity 250 μCi $mmol^{-1}$) to all samples and incubation continued for 3 h. Following incubation, buffer was removed and leaf discs were washed five times for five minutes in excess 25 mM MES pH 5.5, 20 mM $CaCl_2$ in order to remove all apoplastic label. Leaf discs were then surface dried using paper towels, pressed between photographic

paper and dried by lyophilisation. Following drying, leaf discs were exposed to Kodak Biomax X-ray film for 1 week at -80^{o}C prior to film development.

RNA isolation and cDNA synthesis

Total RNA was extracted from flowers and ripening fruit according to the method of Iandolino *et al.* (2004) with modifications described by Reid *et al.* (2006). Plant tissues were ground to a powder in liquid nitrogen and added to a prewarmed buffer (65^{o}C) consisting of 300 mM tris pH 8.0, 25 mM EDTA, 2 mM NaCl, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (w/v) PVPP, 0.05% (w/v) spermidine HCl and 2% (v/v) 2-mercaptoethanol. Buffer to tissue ratios were between 5:1 and 8:1 (v/w). Samples were incubated for 30 min with vigorous vortexing every 5-10 min. Samples were centrifuged (3000 g, 10 min, 4^{o}C) and the supernatant filtered through miracloth after which centrifugation was repeated (13000 g, 10 min, 4^{o}C). The supernatant was combined with an equal volume chloroform:isoamyl alcohol (24:1) and gently shaken. The sample was centrifuged (13000 g, 5 min, 4^{o}C) and the aqueous phase re-extracted against the chloroform:isoamyl alcohol mixture. The aqueous phase was then mixed with 0.1 volumes of 3 M sodium acetate buffer pH 5.2 and 0.6 volumes of isopropanol. Following mixing the samples were stored at -80^{o}C for 30 min and then centrifuged (3500 g, 30 min, 4^{o}C). The sample supernatant was discarded and the pellet resuspended in 1 ml 10 mM tris pH 7.4 containing 1 mM EDTA (TE). 0.3 ml 8 M LiCl was added and RNA precipitated by overnight storage at 4^{o}C. The RNA was collected by centrifugation (16000 g, 30 min, 4^{o}C), washed twice in ice-cold 70% ethanol, air dried and resuspended in 50 to 100 µl RNAse-free water. RNA concentration and purity was estimated by measurement of the optical density at 230, 260 and 280 nm using a nanodrop ND-1000 spectrophotometer. Where considered necessary, RNA extracts were run on formaldehyde agarose gels (1%) to check their integrity. Gels were electrophoresed at 70 V in a buffer containing 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1mM EDTA and 2.5 M formaldehyde and RNA was visualised under ultraviolet light after staining with ethidium bromide (200 ng ml^{-1}).

cDNA was generated from RNA templates by reverse transcription using Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences, UK) according to the manufacturers instructions with pd(N)$_6$ random hexamers (GE Healthcare, UK) acting as primers. The reaction product was used directly as template for subsequent PCR reactions.

Measurement of gene expression

Gene expression was estimated by quantitative PCR using templates generated from RNA as described above. Primers were designed for the amplification of short (~ 100 bp) stretches of cDNA sequence corresponding to genes encoding GDP-mannose pyrophosphorylase (Fig. M1), GDP-mannose 3,5-epimerase (Fig. M2), GDP-L-galactose phosphorylase (Fig. M3) and L-galactose-1-phosphate phosphatase (Fig. M4) and were used at a final concentration of 0.3 μM. Quantitative PCR was performed according to the manufacturers instructions using a Chromo4 Real-Time PCR Detector (Biorad, UK) in the presence of the double stranded DNA binding dye SYBR Green (Quiagen, UK) with DNA amplification monitored by measuring fluorescence emission at 521 nm following excitation at 494 nm between each amplification cycle. Relative gene expression was calculated with the aid of Opticon Monitor 2.03.05 software (MJ Research, MA, USA). Gene amplification was calculated relative to that of 18S rRNA amplified using the primer pair 5'-tgg aag gga cgc att tat ta-3' and 3'-gta gta agt tta aag acg gg-5'. The suitability of all primer combinations was determined prior to quantification of gene expression by determination of the relative efficiency of amplification over a dilution series of 4 orders of magnitude. The absence of primer-dimers was monitored by performing a melting curve following completion of the PCR reaction.

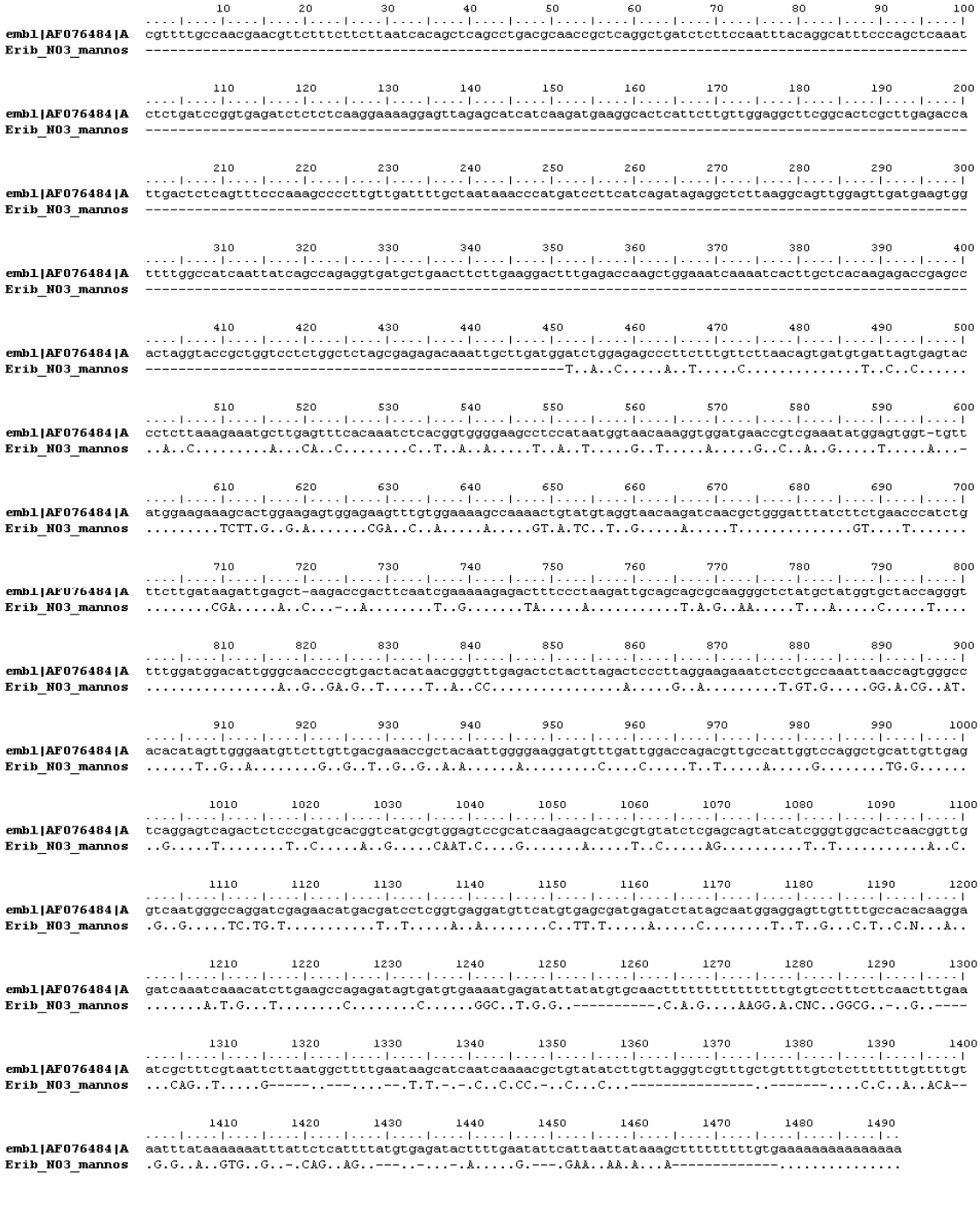


Figure M1 Alignment of putative *R. nigrum* GDP-mannose pyrpohosphorylase with *A. thaliania* GMP1

Putative GDP-mannose pyrophosphorylase from R. nigrum (Erib_NO3_mannos) was cloned as part of an EST library isolated from green/red blackcurrant fruit and identified by conducting a blast search against the EMBL gene library. The alignment with A. thaliana GMP1 (embl[AF076484]A) is shown. Identical residues are represented by a dot and missing residues are represented by a dash. Primers used for qRT-PCR were designed to residues 1060-1079 and 1164-1183 for forward and reverse primers respectively. Two overlapping primer pairs were used in order to produce PCR fragments for sequencing: primer pair 1 were 573-593 (forward) and 990-1009 (reverse) and primer pair 2 were 955-974 (forward) and 1417-1444 (reverse).

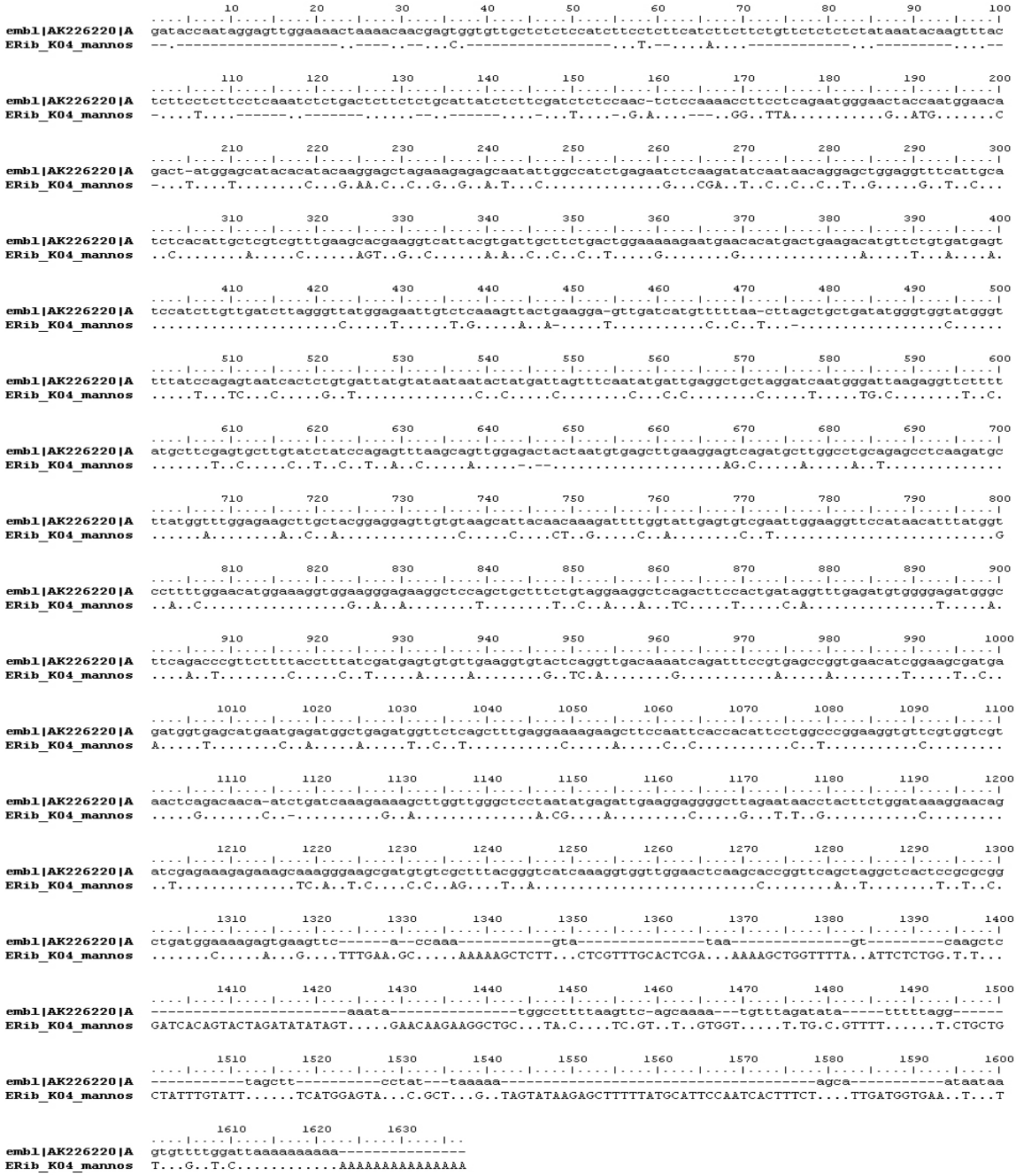


Figure M2 Alignment of putative *R. nigrum* GDP-mannose 3,5-epimerase with *A. thaliania* GDP-mannose 3,5-epimerase

Putative GDP-mannose epimerase (Erib_KO4_mannos) from R. nigrum was cloned as part of an EST library isolated from green/red blackcurrant fruit and identified by conducting a blast search against the EMBL gene library. The alignment with A. thaliana GDP-mannose 3,5-epimerase (embl[AK226220]A) is shown. Identical residues are represented by a dot and missing residues are represented by a dash. Primers used for qRT-PCR were designed to residues 876-895 and 995-1014 for forward and reverse primers respectively. Three overlapping primer pairs were used in order to produce PCR fragments for sequencing: primer pair 1 were 144-169 (forward) and 751-770 (reverse), primer pair 2 were 722-739 (forward) and 1316-1335 (reverse), and primer pair three were 1278-1297 (forward) and 1562-1582 (reverse).

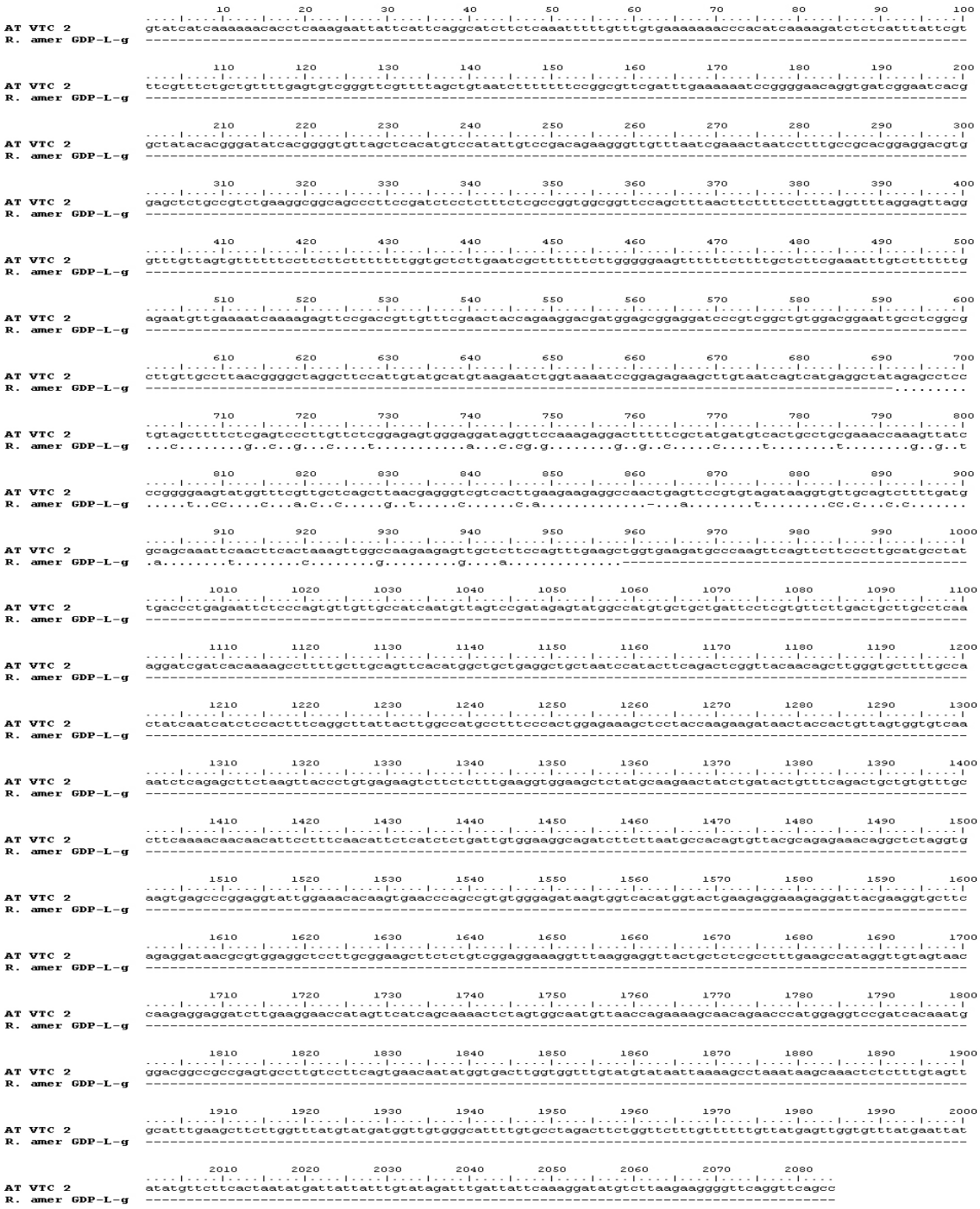


Figure M3 Alignment of putative *R. americanum* GDP-L-galactose phosphorylase with *A. thaliania* VTC2

Putative R .americanum GDP-L-galactose phosphorylase (R. amer GDP-L-g) was identified by performing a blast search using A. thaliana VTC2 (AT VTC 2) to search an EST library generated from young flower buds and inflorescences hosted by the plant genome network at Cornell University (http://pgn.cornell.edu/). Identical residues are represented by a dot and missing residues are represented by a dash. Primers used for qRT-PCR were designed to residues 773-792 and 872-891 for forward and reverse primers respectively.

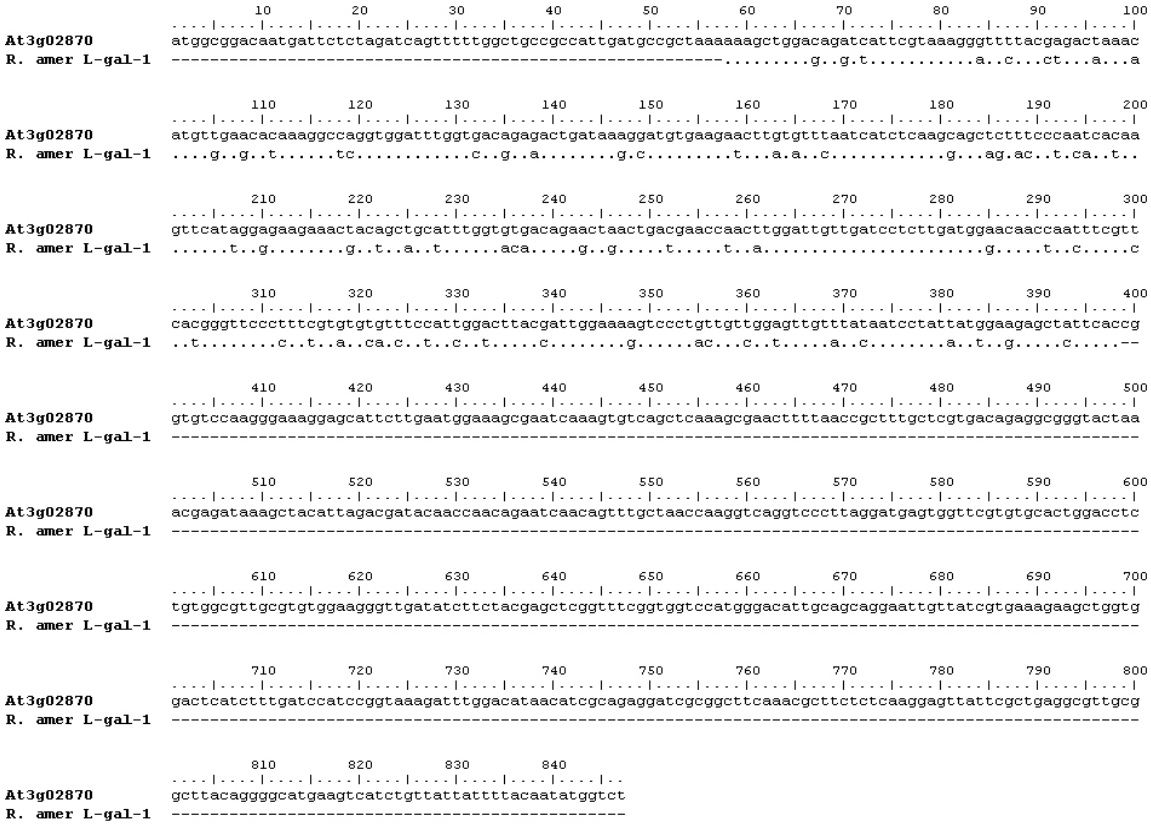


Figure M4 Alignment of putative *R. americanum* L-galactose-1-phosphate phosphatase with *A. thaliania* At3g02870

Putative R .americanum L-galactose-1-phosphate phosphatase (R. amer L-gal-1) was identified by performing a blast search using A. thaliana L-galactose-1-phosphate phosphatase (At3g02870) to search an EST library generated from young flower buds and inflorescences hosted by the plant genome network at Cornell University (http://pgn.cornell.edu/). Identical residues are represented by a dot and missing residues are represented by a dash. Primers used for qRT-PCR were designed to residues 201-219 and 287-306 for forward and reverse primers respectively.

Manipulation of temperature regime

Five plants each of cvs Hedda and Baldwin and genotype 8982-6 were transferred to three different controlled environment chambers at the beginning of March 2006, prior to any visible bud burst. Chambers were set to 70% relative humidity, 16 h/8 h day/night cycle and three temperature regimes were applied; 20^{o}C/15^{o}C, 20^{o}C/12^{o}C and 20^{o}C/8^{o}C during the day and night and respectively. Plants were maintained under these conditions until fruit matured. Fruit were harvested and stored at -80^{o}C until analysis of AsA concentration by HPLC.

Manipulation of nitrogen supply

Year 2 (2003)

Application of different nitrogen concentrations to potted bushes

There is evidence in other crops that high nitrogen application can have a negative effect on ascorbic acid concentration. This may simply be due to excessive vegetative growth shading the developing crop (Lee & Kader, 2000). The effect of nitrogen supply was experimentally tested by applying five different concentrations of nitrogen to potted blackcurrant bushes using standard conventional fertigation technology, while keeping other macro- and micro-nutrients constant.

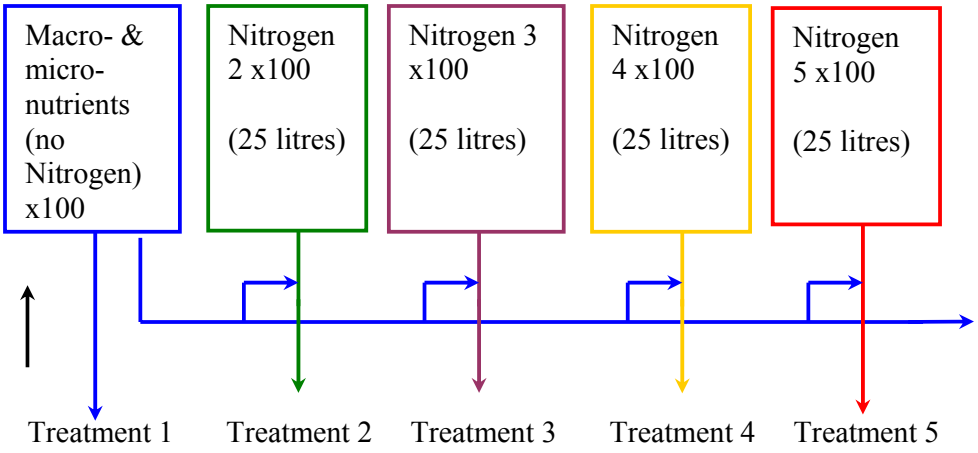


Figure M5 A diagram showing how the stock solution of macro- and micro-nutrients was mixed with incoming mains water. This provided the fertigation for treatment 1 (no nitrogen). In addition, increasing concentrations of ammonium nitrate solutions were diluted 100x into the macro- and micro-nutrient fertigation solution to provide the fertigation solutions for treatments 2, 3, 4 or 5

Two-year-old bushes of the blackcurrant cv. Baldwin were obtained from Hargreaves Plants Ltd, Spalding, Lincs. The bushes were potted in the winter of 2002 into 4-litre pots containing nutrient-free compost (Bulrush Peat Co.) The potted bushes were placed outside on a free draining gravel bed. Also, potted bushes of blackcurrant cv. Hedda were obtained via SCRI from Norway, in May 2002. In order to receive adequate chilling, the potted Hedda bushes were placed in a +2^{o}C dark, humid cold store in January 2003 for 4 weeks. The bushes were then re-potted into nutrient-free compost as for cv. Baldwin, and placed outside on the same gravel bed.

The potted bushes were arranged in a split-plot design: 2 cultivars × 5 nitrogen treatments × 6 replicates, with 2 main plots (cultivars) within each replicate block and 5 sub-plots (nitrogen concentrations) within each main plot. Irrigation was by means of an automatic fertigation system, as shown in Fig. M5.

Table M1 Final concentrations of macro- and micro-nutrients in parts per million (ppm or mg dm^{-3}) in the fertigation solutions

Nutrient	Nutrient concentration delivered daily as mg dm^{-3} (or ppm)
Phosphorus	40
Potassium	156
Magnesium	36
Manganese	0.55
Sodium	34
Boron	0.32
Copper	0.06
Molybdenum	0.48
Iron	2.8
Sulphur	113
Chloride	287
Nitrogen - *treatment 1*	19
Nitrogen - *treatment 2*	131
Nitrogen - *treatment 3*	243
Nitrogen - *treatment 4*	467
Nitrogen - *treatment 5*	915

No calcium was added because analytical results obtained from the local water supply company (Mid Kent Water) indicated that the mean calcium concentration in mains water was 82 mg dm^{-3} (ppm), which was sufficient to supply the calcium needs of the blackcurrant bushes. The nitrogen values for treatments 1-5 in Table M11 represent total nitrogen (ammonium + nitrate), and includes the contribution from nitrate present in the mains water (mean value 19 ppm, according to Mid Kent Water analyses).

Nitrogen fertigation was started on 14 April 2003; 2 × 15 min fertigation per day at 2 litres h^{-1} per pot, thus 1 litre of fertigation solution was applied per pot per day. The pHs of the fertigation solutions, and the pH of the compost, were monitored regularly throughout the experiment. Two leaves were tagged per bush and chlorophyll fluorescence was measured, non-destructively, weekly using a 'chlorophyll content meter' (CCM-200, Opti-Science,

USA). A large proportion of leaf nitrogen is in the form of the photosynthetic pigment chlorophyll, so measurements of chlorophyll content can be related to estimates of leaf nitrogen concentration. Direct validation of the relationship between non-destructive measurements of chlorophyll and extractable leaf protein yielded a good proxy for leaf nitrogen concentrations. In addition, one strig per bush was tagged and flower/fruit number and size were measured at regular intervals throughout the fertigation experiment.

Fruit was harvested from the bushes at the start of July 2003 and graded with respect to colour (green, red or black) and size (diameter <5.6 mm, 5.6-8 mm, 8-11.2mm, >11.2mm) classes. Fresh weights for all size classes were also recorded. Fruit samples were frozen in liquid nitrogen and subsequently stored at -80^{o}C until AsA determination. The blackcurrant bushes were then harvested themselves to determine total leaf area, leaf fresh and dry weight, and dry weights of old shoots (previous season's growth), new shoots (current season's growth), and the amount of root matter. This information was subsequently used to determine dry matter allocation to various plant parts. The nitrogen concentration of ten leaves from each bush was estimated using a 'chlorophyll content meter', and these leaves were then used for commercial mineral analysis (performed by Natural Resource Management Ltd, Bracknell, Berkshire, UK).

Field planting design

Two-year-old bushes of blackcurrant cvs Baldwin and Ben Lomond were delivered on 29/10/02 and kept in moist +2^{o}C cold store until planting on West Egham field on 19/12/02. The bushes were planted in 7 rows containing 180 plants (a group of 90 Baldwin and another group of 90 Ben Lomond). The spacing between rows was 3m and between bushes was 0.5 m. Unfortunately, the establishment of the Ben Lomond plants was so poor that all the plants had to be replaced in November 2003 and therefore they were not used for experimental purposes in 2004.

Year 3 (2004)

Application of different nitrogen concentrations to field-grown bushes

A field experiment was designed to evaluate the impacts of five levels of nitrogen (treatments), which were applied in a four replicate randomised block design across 90

Baldwin bushes. Each treatment was represented once in each of the four blocks. Each replicate plot contained two bushes, with each plot separated by two guard bushes. Each plot is 1m wide. Calcium ammonium nitrate (76 kg N ha^{-1}) was applied during April 2004. Due to an unfortunate error during application, treatments N1 and N2 received the N3 treatment and were therefore excluded from any further analysis during 2004. Calcium ammonium nitrate (76 kg N ha^{-1}) was applied again to treatments N4 and N5 on 10 May 2004 and to treatment N5 only again on 3 and 30 June.

Treatments

1.	N1	No fertiliser	
2.	N2	Half standard application rate	(38 kg N ha^{-1})
3.	N3	Standard application rate	(76 kg N ha^{-1})
4.	N4	Double standard application rate	(152 kg N ha^{-1})
5.	N5	Quadruple standard application rate	(304 kg N ha^{-1})

Leaf nitrogen concentration was estimated non-destructively using a chlorophyll content meter, with measurements made on the 4 and 5 leaf (i.e. fully expanded) on new shoot extension growth using one bush per plot on 18, 26 May, 3, 11, 19, 25 June and 6 July 2004.

The fruit were harvested from all the bushes in treatments N3, N4 and N5 on 19 July 2004. The total yield per bush was recorded. Size distribution was determined by passing a 10-20% sub-sample (selected at random) per bush through sieves. Thus, the fruit were graded into three size classes; >11 mm, 8-11 mm, 5.6-8 mm diameters. The number and weight of berries in each size class was determined. A further sub-sample was taken, for AsA analysis, frozen in liquid nitrogen and stored in freezers at -80^{o}C.

To determine treatment impacts on plant vegetative growth and potential carry-over influences of treatments in to the following year, shoot growth measurements were carried out during January 2005. A count of all new shoots that had grown the previous year and the length of these new shoots were determined. Total shoot growth was determined by multiplying shoot number by the average shoot length.

Year 4 (2005)
Application of different nitrogen concentrations to field-grown bushes

For Baldwin blackcurrant bushes the same experimental setup as in year 3 was used, using the same bushes to apply the same levels of N-treatments. Ben-Lomond bushes were also used in year 4, with a similar setup as with the Baldwin.

On 18 April 2005, nitrogen was applied as calcium ammonium nitrate at a rate of 76 kg N ha^{-1} to N3, N4 and N5. To N2 calcium ammonium nitrate at a rate of 38 kg N ha^{-1}. No nitrogen was applied to N1 treatment. On 19 May a further application calcium ammonium nitrate at a rate of 76 kg N ha^{-1} was applied to N4 and N5. A third application at the same rate was applied to treatments N5 on 15 June. The last application was made on 6 July to the N5 treatment.

The fruit were harvested on 21 July 2005. The total yield per bush was recorded. Size distribution was determined by passing a 10-20% sub-sample (selected at random) per bush through sieves. Thus, the fruit were graded into three classes; >11 mm, 8-11 mm, 5.6-8 mm. The number and weight of berries in each size class was determined. Fruit Brix was determined on different size classes of a sub sample of fruit. A further sub-sample was taken, for AsA analysis, frozen in liquid nitrogen and stored in freezers at -80^{o}C.

Manipulation of crop load

Year 2 (2003)
Effects of strig removal on fruit development and [AsA]

The aim of this experiment was to determine if a reduction in crop load might induce an increase in berry [AsA] at final harvest. A pilot experiment had been performed on blackcurrant cv. Baldwin with two treatments: control and 50% crop thinning, applied just after fruit set. Thinning was achieved by complete flower strig removal, not by thinning within a 'strig' (a short shoot on which flowers and fruit are borne**).** The number and weight of berries per treatment was determined. A sub-sample was taken, for AsA analysis, frozen in liquid nitrogen and stored in freezers at -80^{o}C.

Effects of strig removal on fruit development and [AsA]

A repeat experiment was carried out in 2003 to include less intense thinning (e.g. 20%) than the 50% used in 2002. It was hypothesised that this level of thinning (20%) might not affect final crop yield, as fruit run-off (abscission prior to fruit set) occurs naturally even in the absence of thinning (i.e. on the unthinned controls), and that this level of fruit reduction could influence final [AsA]. Again, as with the previous experiment flower thinning, was carried out by the removal of complete strigs. The treatments were as follows:

Treatments

1. Control (no thinning only natural abscission)
2. 20% thinning
3. 50% thinning
4. 80% thinning

Bushes of the blackcurrant cvs Baldwin and Hedda were obtained as described for the nitrogen experiments above. The bushes were potted into 4-litre pots containing a commercial compost mix with controlled release fertiliser (Osmocote 15:8:10 NPK). The potted bushes were arranged on a free-draining gravel bed in a split-plot design: 2 cultivars × 4 thinning treatments × 6 replicates, with 2 main plots (cultivars) within each replicate block and 4 sub-plots (thinning treatments) within each main plot. The flower thinning treatments were applied on 23 April 2003, when the Baldwin and Hedda bushes were at fruit development stage F3 (100% flowers open). Each bush was individually irrigated using a commercial, automatic drip irrigation system. One strig per bush was tagged and flower/fruit number and size were measured at regular intervals throughout the experiment. Fruit was harvested from the bushes at the start of July 2003. Analyses were performed as for the nitrogen experiment (see above), apart from leaf mineral analysis.

Year 3 (2004)

Effects of fruit removal within a strig on fruit development and [AsA]

A third experiment was designed to explore the hypothesis that controls over fruit development and fruit [AsA] could be regulated at the strig level. Such that a flowering/fruiting shoot (a strig) behaves as an autonomous unit. This experiment on

Baldwin bushes comprised of two treatments in which potential crop load was adjusted by reducing the potential number of berries on each strig.

Treatments

1. C No fruit removal
2. T50 Bottom 50% of set fruit/flowers on every strig removed (thinned)

The treatments were applied (on 12 May 2004 during fruit set) in a 10 replicate complete randomized block. Each plot contained one bush. Each block was separated by a guard bush. The treatment was applied by removing the lower proportion (50%) of berries on all the strigs on a plant. This effectively removed the later developing, or less well developed berries. The fruit were harvested on 22 July 2004 and total harvest yield per bush recorded. A 10-20% sub-sample were size graded using the same method as for the nitrogen manipulation experiment. A further sub-sample for AsA analysis was frozen in liquid nitrogen and stored frozen at -80^{o}C. Shoot growth measurements were carried out during January 2005. A count of all new shoots that had grown the previous year and the length of these new shoots were determined.

Year 3 (2004)

Description of natural variability in strig berry number and [AsA]

A complementary experiment was carried out to determine if natural differences in fruit set (natural variation in strig berry number) may contribute to difference in berry development and [AsA]. To test this hypothesis eight previously unused bushes were selected at random. From each bush five large strigs containing 8-14 berries and five small strigs containing 2-5 berries were removed. Only nodes containing single strigs were selected. The strigs were removed on the 1 July 2004. Berry diameter was measured using callipers and maturity based on berry colour was recorded for each. The berries were grouped separately for large and small strigs for each of the bushes according to size classes <5.6mm, 5.6-8mm, 8-11.2mm and >11.2mm diameter. Each sample was frozen in liquid nitrogen and frozen at -80^{o}C for later AsA analysis.

Year 4 (2005)

Effects of source manipulation by strig leaf removal and branch girdling

The aim of this experiment was to determine the effects of different resource supplies (photoassimilates compared with bark-stored carbohydrates) on seasonal production of AsA. Photoassimilate supply was manipulated by strig leaf removal in late May, while the potential for carbohydrate supply to be derived from stored reserves was investigated by branch girdling to remove translocation from other branches, leaves or roots.

Treatments

1. Strig leaf removal
2. Branch base girdling

On 25 May, 30 bushes of Ben Tieran were selected within two rows and the strig leaves removed. On each bush three branches were stripped of all strig leaves, leaving intact the terminal leaves. A further three branches were selected where the strig leaves were untouched to act as controls. The bushes were grouped in pairs and the branches on one of the pair of bushes were girdled. This was achieved by 'circumcising' the bark as close to the branch base as possible. Flowers were removed from elsewhere on the bush, frozen in liquid nitrogen and stored at -80^{o}C for AsA analysis.

On 16 June green fruit were harvested, removing fruit from one of each of the branches with and without strig leaves, for all the 30 bushes. Fruit were graded into 3 size categories, counted, weighed and a sample frozen in liquid nitrogen and stored at -80^{o}C for AsA analysis.

On 11 July the procedure described above was repeated to collect another sample of fruit (green), but only for non-girdled bushes as the majority of branches that had been girdled had died. Again fruit were graded into three size categories, counted, weighed and a sample frozen in liquid nitrogen and stored at -80^{o}C AsA analysis.

Year (2006)

Effects of age of wood on strig berry number

The aim of this experiment was to determine the variation in fruit yield, [AsA] and AsA yield with bush wood age. Our hypothesis is that innate difference in fruit yield and [AsA] may be related to the age of wood (1-year-old wood, 2-year-old wood, etc.) on which

flowers and fruit are borne. To test this hypothesis, branches on 10 bushes of Baldwin and 10 bushes of Ben Lomond were labelled in the spring of 2006. The number of strigs and number of fruit per strig were assessed on different aged wood on 15 May, 14 June and 27 July 2006. At harvest on 27 July the fruit from the different aged wood was collected separately. The fruit were size graded and frozen in liquid nitrogen and stored at -80^{o}C for subsequent chemical analysis.

Year 5 (2006)

Determination of the impact of autumn leaf defoliation on fruit [AsA] in the subsequent growing season

Our aim was to determine what impact the presence of leaves (post-harvest) might have on fruit [AsA] in the following season. The hypothesis tested was that the photosynthetic carbon contribution of leaves, via reserve carbohydrates, directly after harvest and prior to leaf fall, was an important contribution to the subsequent season's fruit growth and fruit [AsA]. To test this hypothesis we have used bushes that during the summer of 2005 have been subject to early leaf defoliation due to leaf spot (*Drepanopeziza ribis)* infection (from a plot belonging to Dr Angela Berrie). From collections of fruitlets early in the 2006 season we suggest that knowledge of fruit [AsA] could enable questions about the importance of leaves and storage carbohydrate to be rapidly addressed (*Experiment 1*). A further experiment was initiated in 2006, where leaf defoliation was carried out manually using bushes that were free from leaf spot infection (*Experiment 2*). This experiment will not yield results this year, but will avoid possible experimentally confounding issues that might arise in response to leaf spot infection.

Experiment 1. Impact of early defoliation by Drepanopeziza ribis (leaf spot), on subsequent fruit [AsA]

Treatments

1. Early Leaf defoliation caused by leaf spot infection in 2005 and sampled in 2006
2. Control - Leaf defoliation at the end of normal growing season 2005 (natural defoliation) and sampled in 2006

This experiment was conducted on Baldwin and Ben Lomond bushes. The early leaf defoliation bushes were on a plot at EMR that was not sprayed against leaf spot disease in 2005, complete leaf defoliation occurred by around the end of August 2005 for Baldwin and Ben Lomond. The bushes on these plots were planted in March 2002. The control bushes of Baldwin and Ben Lomond were on a plot that had not undergone early defoliation; these bushes were planted in winter 2002 and 2003 respectively and were therefore a least 2-years-old and were of similar size. Due to the controls and the leaf defoliation bushes being on different plots the experiment could not be fully replicated in a randomised block design. A further leaf defoliation experiment has been initiated in 2006 for fruit collection in 2007 is of a complete randomised block design (see below).

Blackcurrant berry samples were taken from 1-year-old wood of bushes on 16 May, 12 June and 25 July 2006. The fruit were size graded into 5.6-8mm, 8-11mm and 11mm+, for all dates except the 16 May where the fruit were all of a small size below 5.6mm, and frozen in liquid nitrogen and stored at -80^{o}C for subsequent AsA analysis.

Experiment 2. Impact of post harvest leaf removal on subsequent fruit [AsA]

The aim of this experiment was to terminate potential post harvest bush photosynthesis, and reduce the possibility for extensive bark carbohydrate storage during late summer and the autumn, by manual leaf removal. The experimental hypothesis was therefore similar to that described above, but will not yield results on fruit [AsA] until harvest the following year. To achieve this aim the time of leaf fall was artificially determined by stripping leaves from entire Baldwin bushes. The analysis of fruit yield, [AsA] and AsA yield was subsequently determine the following year (i.e., 2007).

Treatments

1. Control – no leaf removal
2. Leaf removal directly after harvest
3. Leaf removal one month after harvest
4. Leaf removal two months after harvest
5. Leaf removal three months after harvest

The treatments were applied in a 10 replicate complete randomized block design, with one bush per treatment per block. All leaves were removed from bushes at different times firstly, at fruit harvest on 2 August 2006 (Treatment 2), then '1 month' after harvest on the 4 September 2006 (Treatment 3), 2 months after harvest the 9 October 2006 (Treatment 4) and the '3 month' treatment on the 27 October 2006 (Treatment 5). The carry-over effect of leaf removal on fruit yield and [AsA] and AsA yield will be determined following year. The effects on shoot growth have been determined during the dormant season.

The fruit were harvested from all the bushes on 18 July 2007. The total yield per bush was recorded. Size distribution was determined by passing a sub-sample (selected at random) per bush through sieves. The fruit were graded into 3 classes; >11 mm, 8-11 mm, 5.6-8 mm. The weight of berries in each size class was determined. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Year 5 (2006)

Determination of the impact of flower removal, in spring, on fruit [AsA] in the subsequent growing season

The aim of this experiment was to determine the impact that flower removal, in spring, has on fruit development and fruit [AsA] the following year. Our hypothesis is that the early fruit development acts as an important sink for carbohydrate and that flowering may compete with the substrates required for the biosynthesis of AsA in leaves and fruit. The fruit's sink activity may therefore be a more important factor in influencing fruit growth and AsA accumulation rather than storage carbohydrate in the bark etc (see above). Early flower removal could potentially restrict carbohydrate loss incurred at fruit harvest and promote carbohydrate storage (bark) for the following season. The analysis of fruit yield and [AsA] was carried out will determine the impacts of removing this sink (flowers) on fruit yield, [AsA] and AsA yield on the cv. Baldwin in the subsequent year.

Treatments

1. Control- no flowers removed
2. All flower removed from a bush

The treatments were applied in a ten replicate complete randomized block design, with one bush per treatment per block. Flowers were removed from treatment 2 on the 8 May 2006. The effect this has on fruit yield, [AsA] and AsA yield was determined in 2007. The effects on shoot growth have been determined during the dormant season.

The fruit were harvested from all the bushes on 18 July 2007. The total yield per bush was recorded. Size distribution was determined by passing a sub-sample (selected at random) per bush through sieves. The fruit were graded into 3 classes; >11 mm, 8-11 mm, 5.6-8 mm. The weight of berries in each size class was determined. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Year 5 (2006)
Determination of the impact of age of bush bearing wood on fruit [AsA]

The aim of this experiment was to determine the variation in fruit yield, [AsA] and AsA yield with bush wood age. Our hypothesis is that innate difference in fruit yield and [AsA] may be related to the age of wood (1-year-old wood, 2-year-old wood etc.) on which flowers and fruit are borne. To test this hypothesis, branches on 10 bushes of Baldwin and 10 bushes of Ben Lomond were labelled in the spring of 2006. The number of strigs and number of fruit per strig were assessed on different aged wood on the 15 May, 14 June and 27 July 2006. At harvest on the 27 July the fruit from the different aged wood was collected separately. The fruit were size graded and frozen in liquid nitrogen and stored at -80^{o}C for subsequent chemical analysis.

Manipulation of water supply

Year 2 (2003)
Effects of differences in irrigation on fruit development and [AsA] in potted bushes

It was hypothesised that early application of partial root irrigation (PRI), based on published knowledge of how early berry [AsA] is determined, was more likely to increase or maintain [AsA]. It may also be less likely to have a negative affect final crop yield, than continuous or late application of partial root irrigation. Blackcurrant bushes were potted into a dual split-pot arrangement, so that approximately 50% of the plant's root system was

in each pot. This enabled imposition of partial root drying (PRI), whereby one of the pots is watered using drip irrigation, and the other pot is allowed to dry. Pots were covered with polythene to prevent precipitation directly entering the pots and influencing the results. Under PRI, the bush receives sufficient water, but the roots in the dry pot will generate stress signals, which can influence various physiological and biochemical processes, including the synthesis of compounds such as AsA. While PRI is being applied, the dry and irrigated pots of any one bush are changed over regularly, so that soil drying does not become excessive and kill the dry part of the root system.

Treatments

1. Control (no PRI, both pots irrigated)
2. Continuous (PRI applied throughout the experiment)
3. Early (PRI applied for the first 6 weeks of the experiment, and then both pots irrigated thereafter)
4. Late (both pots watered for the first 6 weeks of the experiment, and then PRI applied thereafter)

The early application of PRI was carried out at the same time as the flower strig thinning. At this stage very little fruit development would have taken place. However, the PRI treatment applied in the latter part of the season would have been at the stage of well-formed fruit.

Bushes of blackcurrant cvs Baldwin and Hedda were obtained as described for the nitrogen experiments above. The bushes were potted into a commercial compost mix with controlled release fertiliser (Osmocote 15:8:10 NPK). The potted bushes were arranged on a free-draining gravel bed in a split-plot design: 2 cultivars × 4 PRI treatments × 6 replicates, with 2 main plots (cultivars) within each replicate block and 4 sub-plots (PRI treatments) within each main plot. The PRI treatments were applied on 24 April 2003, when the Baldwin and Hedda bushes were at fruit development stage F3 (100% flowers open). Treatments were applied by removing irrigation drippers from pots as appropriate. After 6 weeks, irrigation was restored to both pots for treatment 3 (early PRI), and removed from one of the pots for treatment 4 (late PRI). Compost drying was measured at regular intervals throughout the experiment with *in situ* soil tensiometers. The switch over generally occurred around two weekly intervals, when the pot with the drying compost was

showing a negative tension >-500 HPa. One strig per bush was tagged and flower/fruit number and size were measured at regular intervals throughout the experiment. Fruit was harvested from the bushes early July 2003. Analyses were performed as for the nitrogen experiment (see above), apart from leaf mineral analysis.

Year 3 (2004)

Effects of differences in irrigation on fruit development and [AsA] in field-grown bushes

This field experiment comprised of three treatments in which irrigation was adjusted to induce partial root drying and maintain soil near field capacity.

Treatments

1. None No irrigation
2. PRD Irrigation alternated between each side of the bush
3. Full Irrigation applied on both sides of bush

The treatments were applied in seven replicate complete randomized blocks. Each experimental plot contained three bushes. Each plot was separated from the next plot by a single guard bush. Black plastic mulch was along the full row (i.e. including Baldwin and Ben Lomond) on 14 April 2004. However, it was only Baldwin bushes that the treatments were applied to as Ben Lomond were not planted until November 2003. The plastic extended for 1m from either side of the bush. The external edge of the plastic was buried into the soil. Irrigation was applied via boot lace drippers, positioned 10 cm from the base of each bush in each plot, and pushed through the plastic mulch. Water was applied three times a day for 10 min with 2 l hr^{-1} emitters from 14 May 2004. For the PRI treatment, the irrigation was switched from the East side of the bushes to the West side on 28 May 2004. This approach was used to allow part of the root system to dry out and mimic soil drying via partial root drying. All irrigation was switched off in mid-September.

To determine the effectiveness of the drought treatments, soil water potential (closely related to soil moisture content) was measured using tensiometers. A tensiometer was installed, 13 cm perpendicular from base on the East side of the middle bush in each plot to a depth of 20 cm on 7 May 2004. Another tensiometer was placed on the West side of the middle bush in each of the PRI treatment plots. Single tensiometers were placed in similar

positions next to seven Baldwin bushes in row 5 to allow comparison with soil where rainfall was not excluded by the plastic mulch. Soil water potential was recorded on 13, 20, 26 May and 4, 15, 28 June 2004.

To determine any differences in soil temperature caused by the plastic mulch and perhaps the different irrigation treatments, a soil temperature probe was placed at a distance of 15cm from the middle bush of each plot, to a depth of 10 cm. Temperature was recorded every 10 min and hourly means were logged using a data logger (Delta-T Devices, Cambridge, UK). The fruit were harvested and on 20 and 21 July 2004. The fruit were graded using the same procedure as for all the other experiments. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C. Shoot growth measurements were carried out during January 2005. A count of all new shoots that had grown during 2004 was determined, along with the length of these new shoots.

Year 4 (2005)

Effects of differences in irrigation on fruit development and [AsA] in field-grown bushes

For Baldwin blackcurrant bushes the same experimental setup as in year 3 was used, using the same bushes to apply the same levels of irrigation. Ben-Lomond bushes were also used in year 4, with a similar setup as with the Baldwin.

Irrigation was applied *via* boot lace drippers, positioned 10 cm from the base of each bush in each plot, and pushed through the plastic mulch. Water was applied three times a day for 15 min (Baldwin) and 6 min (Ben Lomond) with 2 litres per h emitters from April 22 2005. To determine the effectiveness of the drought treatments, soil water potential (closely related to soil moisture content) was measured using tensiometers for Ben Lomond. A tensiometer was installed, 13 cm perpendicular from base on the East side of the middle bush in each plot to a depth of 20cm on 12 and 13 May 2005. Another tensiometer was placed on the West side of the middle bush in each of the PRI treatment plots. Soil water potential was recorded on occasion throughout the season. For Baldwin, Theta probes were used to record soil moisture content, these were buried in the soil to a depth of 20cm, placed on the East side of the middle bush in each plot, of the 3 middle blocks, at a distance of 13cm from the bush. An additional theta probe for PRI was placed on the West side. Readings were logged on a Delta-T logger daily.

The Baldwin fruits were harvested on 26 July and graded using the same procedure as for the other experiments, while Ben Lomond was harvested on 9 August. Fruit Brix was determined on a sub sample of fruit. A sub-sample of each size class was taken for AsA analysis and frozen in liquid nitrogen and stored at -80^{o}C.

Manipulation of light interception

Year 3 (2004)
Effects of enhanced solar radiation interception using reflective mulches

The direct importance of solar radiation on fruit [AsA] is not clearly understood. The hypothesis of this work is therefore to determine if by manipulating solar radiation interception by bushes can [AsA] increase as well as perhaps yield of fruit. To achieve this light interception was enhanced, to different degrees, by the use of reflective plastic soil mulches. The experiment used two different types of reflective mulches to influence incident radiation interception by the crop canopy and subsequently the effect on crop yield and AsA production were determined.

Treatments

1.	None	Bare soil
2.	'Extenday^{TM}'	Reflective plastic weave cloth (supplied by FAST Ltd, Faversham Kent)
3.	'Solarmat'	Solarisation matting (supplied by Capatex Agro-textiles, Nottingham)

The reflective mulches were laid along the row on 22 April 2004. The 'Extenday' was laid in 1.25 m strips on either side of each bush. The ground was raised to produce a slope that peaked 50 cm from either side of each bush. This directed the reflected light back towards the bushes. A similar procedure was used for installing the 'Solarmat'. The treatments were applied in seven replicate complete randomized blocks. Each experimental plot contained three bushes. Each plot was separated from the next plot by a single guard bush.

Incident light and that reflected by the mulches and bare soil was determined using arrays of photosynthetic active radiation (PAR) sensors. Two PAR sensors were placed 50 cm above the ground adjacent to the middle bush in three plots of each treatment. One sensor

faced towards the ground and the other towards the sky. In addition, three more sensors were placed (1 m height) above the bushes in each treatment respectively to intercept all incoming radiation. Air temperature within the bush was recorded at 15 and 30 cm above the ground and soil temperature was recoded at a depth of 10 cm in the same plots as above. Temperature and PAR were recorded every 10 min and logged hourly using a data logger (Delta-T Devices, Cambridge, UK) from 17 May until 27 July 2004.

The fruit were harvested on 14-16 July 2004 and graded using the same procedure as for all the other experiments. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C. Shoot growth measurements were carried out during January 2005. A count of all new shoots that had grown the previous year and the length of these new shoots were determined.

Year 4 (2005)

Effects of enhanced solar radiation interception using reflective mulches

For Baldwin blackcurrant bushes the same experimental setup as in year 3 was used, with the mulches having been applied on 22 April 2004.

On 28 March 2005, reflective mulch was put down on the Ben Lomond experimental plants as described above (year 3). The treatments were applied in seven replicate complete randomized blocks. Each experimental plot contained three bushes. Each plot was separated from the next plot by a single guard bush.

Incident light and that reflected by the mulches and bare soil was determined using photosynthetic active radiation (PAR) sensors. Two PAR sensors were placed 50 cm above the ground adjacent to the middle bush in three plots of each treatment. One sensor faced towards the ground and the other towards the sky. In addition, three more sensors were placed (1 m height) above the bushes in each treatment respectively to intercept all incoming radiation. Air temperature within the bush was recorded at 15 and 30 cm above the ground and soil temperature was recoded at a depth of 10cm in the same plots as above. Temperature and PAR were recorded every 10 min using a Delta-T logger during the growing season.

The fruit were harvested on 19 for the Baldwin and 2 August for Ben Lomond. The fruit were graded using the same procedure as for the other experiments. Fruit Brix was

determined on different size classes of a sub sample of fruit. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Enhancing [AsA] through exogenous applications of AsA precursors

Year 3 (2004)
Field application of potential AsA biosynthetic precursors

This work was intended to respond to consortium raised issues that precursors to AsA biosynthesis might be a useful tool in enhance [AsA] in berries. L-galactono-1,4(γ)-lactone is currently believed to be the precursor prior to the synthesis of AsA and its conversion to AsA is due to the action of the enzyme L-galactose dehydrogenase (see Conklin, 2001). Our hypothesis is that AsA synthesis may be limited by the availability of these precursors in plant tissues (perhaps leaves).

Three chemicals were applied directly to plants in the field via hand spray applications. Of these only one can be truly considered as a likely direct precursors of AsA synthesis, i.e. L-galactono-1,4(γ)-lactone. This precursor is currently believed to be the final intermediate before L-ascorbic acid (AsA). All applications used a surfactant to maximise potential uptake and sprayed were applied to run-off (around 500ml per treatment).

Treatments

1. W Water (1 ml 'Tween' l^{-1})
2. Su Sucrose (25 mM with 1 ml 'Tween' l^{-1})
3. Gl Glucose (25 mM with 1 ml 'Tween' l^{-1})
4. Ga L-galactono-1,4(γ)-lactone (Galactonic acid; 25 mM with 1 ml 'Tween' l^{-1})

Four branches with good extension growth were selected and tagged on 20 bushes. A single treatment was applied to one of each of these branches on all of the bushes (i.e. 20 branches per treatment) on 6 July 2004. The fruit of 10 bushes were harvested on 7 July 2004. The fruit from each branch (i.e. treatment) were removed separately. The treatments were applied again to the remaining ten bushes on 7 July 2004 and the fruit harvested on 8 July 2004. Fruit were also divided into two groups, those from the top and the bottom of the branch. Size grading was restricted to two classes >11 mm and 8–11 mm. The weight of fruit in each size grade was recorded. A sub-sample for later AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Non-destructive method to determine* in situ *[AsA]

Year 3 (2004)

Berry analysis of AsA using micro-dialysis

An ability to measure *in situ* [AsA], in as non-destructive manner as possible, would enable a number of key experimental questions to be addressed regarding the biosynthesis and the transport of AsA. To achieve this a series of validation experiments were carried out using micro-dialysis (Microbiotech, Stockholm, Sweden) with both detached fruit and potted fruiting bushes growing under controlled environmental conditions (Fig. M6). The use of the micro-dialysis probes followed the basic outline provided by the manufacturers. Probes (MAB 6) were used with a polyether sulphone membrane cut-off of 15,000 Dalton's with a membrane diameter of 0.6mm. Micro-dialysis enables *in situ* measurement of [AsA] directly in attached berries. As far as we are aware this technique has not been used on blackcurrants so initially, we carried out some preliminary validation experiments to determine the best approach to use with the probes and the accuracy and precision with which they worked. Our subsequent aim was to develop the technique to determine localised application of precursors such as L-galactono-1,4-lactone, might influence [AsA] directly over a short period of time.

Figure M6 A diagrammatic illustration of the micro-dialysis (MAB 6) probe Microbiotech, Sweden (left) and an image of a probe mounted in an attached berry with perfusion solution being supplied to the fruit via the upper tube and the resultant exudate from the from being collected via the lower tube (right)

A series of preliminary experiments, with detached fruit, were conducted to evaluate the potential to use micro-dialysis probes (Microbiotech, Luton) to extract AsA non-

destructively from blackcurrant fruit. Initial experiments involved determining the ability of the probes to extract AsA from single fruit (cv. Ben Connan) and the most appropriate profusion times to achieve stable measurements of concentration. All these experiments were conducted either in controlled temperature rooms or controlled environment cabinets.

Manipulation of bush phosphorus concentration to enhance [AsA]

Year 3 (2004)

This experiment determined the effect of the application of a propriety phosphorus/calcium liquid foliar feed (Seniphos, Phosyn plc) under commercial conditions. Seniphos is described by Phosyn "for the improvement of firmness, storage potential and red colouration of fruit crops". Chemicals were donated by Phosyn through Mike Stoker. Previous unpublished work carried out by Phosyn plc, on a grower's farm (Charles Gaskain), suggested that Seniphos application had dramatically increased AsA yield. Despite contacting both Phosyn (staff past and present, i.e. Maurice Banwell) and Charles Gaskain the data relating to this trial has not been forthcoming.

A preliminary experiment was undertaken using two sites growing cv. Ben Hope, sites were selected with assistance from Rob Saunders (GSK) at the farms of Ian and Nick Overy of Mile Oak Farm, Paddock Wood, Kent. One in Horsmonden, Kent had high soil P concentration (51 mg P l^{-1}), whereas the other in Matfield, Kent, had a low concentration (22 mg P l^{-1}). The aim of this experiment was to determine the need for a more detailed designed subsequent experiment. The treatments used were as follows:

Treatments

1. O No application of phosphorus
2. S Application of phosphorus ('Seniphos')

Seniphos [310 g l^{-1} (%) P_2O_5; 40 g l^{-1} (4%) Ca and 39 g 1^{-1} (3.9%) N] was applied using standard industry recommendation rate for Top Fruit of 10 litres per hectare (in 1000 litres), on five occasion between May and July (applications were made as part of a mix applied during standard field sprays). Each site was split into two, one half received supplementary P nutrition, through the application of Seniphos, and the other did not. The

treatments were applied at standard (Phosyn recommended) rates similar to those applied to apples and to those believed to have been applied during the Gaskain's experiment.

Fruit were harvested, by hand, from the Horsmonden site on 21 July and from the Matfield site on 29 July 2004. Ten bushes that had received no application of Seniphos were chosen at random and a further 10 that had received the Seniphos treatment were chosen on each site. Each sample came form a different row and bush and sample rows were only used in the middle of the spray blocks. Complete strigs (~200g samples) were removed from each bush at a similar height and orientation of the bush and subsequently size graded. The weight of fruit in each size grade was recorded. A sub-sample of berries was used to determine soluble sugar concentration and further sub-sample for later AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Year 4 (2005)

This experiment determined the effect of the application of a propriety phosphorus foliar feed (*Seniphos*™, Phosyn plc) under commercial conditions. Chemicals were donated by Phosyn through Mike Stoker. Two sites used were as selected in 2004 (Horsmonden and Matfield) with assistance from Rob Saunders (GSK) at the farms of Ian and Nick Overy of Mile Oak Farm, Paddock Wood, Kent. One in Horsmonden had high P concentration in the soil (51 mg P l^{-1}), whereas the other in Matfield had a low concentration (22 mg P l^{-1}). The treatments were similar to in 2004 with the exception that part of the area treated was left untreated while the other half received an application of Seniphos in 2005 as well as 2004. On both sites the cv. Ben Hope was used for the experimental treatments.

Treatments

1.	O	No application of Seniphos
2.	S+04	Application of Seniphos [31% P_2O_5: 4% Ca and 3.9% N] using standard industry recommendation rate of 10 litres per hectare, during May and July in 2004
3.	S+04 & 05	Application of Seniphos [31% P_2O_5: 4% Ca and 3.9% N] using standard industry recommendation rate of 10 litres per hectare, during May and July in only 2005

Each site was split into two, one half received supplementary P nutrition and the other did not. The treatments were applied at standard (recommended) rates by the owner on 25 May, 9 June and 24 June for Matfield and Horsmonden sites.

Fruit were harvested from the Horsmonden and Matfield sites on 29 July 2005. Ten bushes that had received no application of P in 2004 were chosen at random and a further 10 that had received the P treatment in 2004 and 2005 were chosen at each site. Complete strigs (~200g samples) were removed from each bush at a similar height and orientation of the bush and subsequently size graded, as for the nitrogen manipulation experiment. A sub-sample of fruit was used to determine soluble sugar content and further sub-sample for later AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Year 4 (2005)

Manipulation of bush potassium phosphite concentration to enhance fruit [AsA]

This experiment determined the effect of the application of a propriety Phosphorus and Potassium foliar feed (Farm-Fos-44, Farm-Fos limited) under experimental conditions and repeated application, for Baldwin and Ben Lomond

Treatments

1. None, No application of Farm-Fos-44
2. E, Early application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard industry recommendation rate of 10 litres ha^{-1}
3. EM, Early and mid application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard industry recommendation rate of 10 litres ha^{-1}
4. Mid, Mid application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard industry recommendation rate of 10 litres ha^{-1}
5. ML, Mid and late application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard industry recommendation rate of 10 litres ha^{-1}
6. All, early, mid and late application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard industry recommendation rate of 10 litres ha^{-1}

The early treatment was applied on the 18 May, the mid on the 21 June and the late on the 22 July. The application rate of Farm-Fos-44 was 1000 litres ha^{-1} at a concentration of 1%

(v/v), or around 275 ml bush^{-1}. The Ben Lomond fruit were harvested on 10 August and Baldwin on the 26 July August. The fruit were graded using the same procedure as for the other experiments. Fruit Brix was determined on a sub sample of fruit. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Year 5 (2006)
Manipulation of potassium phosphate effects on fruit vitamin C.

The experimental setup was as year 4, but on a different set of bushes.

The early treatment was applied on the 2 June, the mid on the 30 June and the late on the 20 July. The application rate of Farm-Fos-44 was 1,000 litres ha^{-1} (when diluted) at a concentration of 1% (v/v), or around 275 ml per bush. The fruit were harvested from all the bushes on 24 July 2006 for Ben Lomond and the 27 July for Baldwin. The total yield per bush was recorded. Berry size distribution was determined by passing a sub-sample (selected at random) of fruit from each bush through sieves. The fruit were graded into three classes; >11 mm, 8-11 mm, 5.6-8 mm. The weight of berries in each size class was determined. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C. A further sub-sample for Brix and acidity determinations was frozen at -20^{o}C. The value of measuring shoot growth on these experiments is currently being determined.

Year 5 (2006)
Carry over effect potassium phosphite application on fruit [AsA]

The aim of this experiment was to determine the potential carry over effects of previous season's applications of the following seasons fruit [AsA]. The treatments, a propriety potassium phosphite foliar feed (Farm-Fos-44, Farm-Fos Limited), was applied as described in 2005 on a set of field-grown plants of Baldwin and Ben Lomond.

Treatments (as in 2005)

The fruit were harvested from all the bushes on 19 July 2006 for Ben Lomond and the 26 July for Baldwin. The total yield per bush was recorded. Berry size distribution was determined by passing a sub-sample (selected at random) of fruit from each bush through sieves. The fruit were graded into three classes; >11 mm, 8-11 mm, 5.6-8 mm. The weight

of berries in each size class was determined. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C. A further sub sample for Brix and acidity determinations was frozen at -20^{o}C.

Multi-application treatment – irrigation and reflective mulch

Year 5 (2006)

A major aim of this project was to deliver insights into ways in which growers could optimise current bush management procedures to ensure maximal yields of AsA. To achieve this we initially determined the impact of a number of factors which were likely to contribute to fruit yield and impact of [AsA]. The most promising agronomic factors were included into a multi-combination (2x2 factorial) treatment experiment to determine their combined impact on fruit AsA yields. To achieve this, light reflective mulches were used in combination with supplementary irrigation and their impact on fruit yield, [AsA] and AsA yield have been determined.

Treatments

1. No Mulch and no irrigation (control)
2. No Mulch with irrigation
3. Mulch without irrigation
4. Mulch plus irrigation

The reflective mulch used was 'Extenday^{TM}' (reflective plastic supplied by FAST Ltd, Faversham, Kent); this was laid in 1.25 m strips on either side of each bush on 20 April 2006. The ground was raised at a distance of 50 cm from the bushes along the entire length of the row on either side of the row. The reflective mulch was applied directly to this contoured soil base. The concave curvature of the soil base, in which the bush sat at the bottom, enabled light to be reflected back into the bush. Water was applied via timed trickle irrigation using boot lace drippers, positioned 10 cm from the base of each bush, in each plot. Water was applied three times a day for 5 min with 2 litres per hour emitters from 4 May 2006 until 9 June 2006 when the time of each individual irrigation event was increased to 15 min.

These treatments were applied to bushes of Baldwin and Ben Lomond in four replicate complete randomized blocks. Each experimental plot contained three bushes. Each plot

was separated from the next plot by a single guard bush. Theta probes were used to record soil moisture content (m^3 m^{-3}), these were buried in the soil to a depth of 20cm, placed on the east side of the middle bush in each plot, of 2 blocks per cultivar, at a distance of 13cm from the bush. Readings were recorded and logged daily (Delta-T Devices, Cambridge, UK).

The fruit were harvested from all the bushes on 18 July 2006 for Baldwin and the 19 July for Ben Lomond. The total yield per bush was recorded. Size distribution was determined by passing a sub-sample (selected at random) per bush through sieves. The fruit were graded into 3 classes; >11 mm, 8-11 mm, 5.6-8 mm. The weight of berries in each size class was determined. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C. A further sub-sample for Brix and acidity determinations was frozen at -20^{o}C. The effects on shoot growth will be determined during the dormant season.

Multi-application treatment – Continuation of irrigation and reflective mulch treatments that were started in 2006

Year 6 (2007)

The same experimental treatments as in 2006 were applied to the same experimental bushes in 2007. The reflective mulches remained in-situ over the summer and winter 2006. Water was applied three times a day for 10 min with 2 litres per hour emitters from 20 April 2007.

The fruit were harvested from all the bushes on 23 July 2007 for Baldwin and 19 July for Ben Lomond. The total yield per bush was recorded. Size distribution was determined by passing a sub-sample (selected at random) per bush through sieves. The fruit were graded into three classes; >11 mm, 8-11 mm, 5.6-8 mm. The weight of berries in each size class was determined. A sub-sample for AsA analysis was frozen in liquid nitrogen and stored at -80^{o}C.

Experimental design and statistical analyses

All experimental designs and analysis approaches were approved through consultation with EMR's consultant biometrician (Mrs G Arnold). For all experiments treatment and cultivar effects were examined by analysis of variance (ANOVA), using the statistical programme *Genstat*. Percentage data were subjected to angular transformation before analysis of variance. Percentage data reported in the tables within the Results Section are the original, untransformed data, and the *P* values relate to the transformed data. The standard error of differences (SED) between the means, the residual degrees of freedom (*d.f.*) for comparison P (or *F Prob*.) probabilities are quoted as appropriate with statistically analysed data sets. Least significant differences (LSD) are shown again as appropriate of figures.

RESULTS AND DISCUSSION

Objective 1 - Determine how, where and when AsA is made in the plant

Rationale

At the start of the current project significant knowledge was available regarding the mechanism of biosynthesis and function of AsA in photosynthetic tissues, however little was known regarding its mechanism of accumulation or functions in non-photosynthetic sink tissues. The aims of objective 1 were therefore to develop a clear understanding of mechanism of AsA accumulation in blackcurrant fruit and the factors that limited accumulation. It was intended that the knowledge gained could be used to guide agronomic experiments and define targets for the identification of markers in seedlings that would allow prediction of fruit AsA concentration in mature plants allowing the development of accelerated breeding programmes.

Three hypotheses were considered for the accumulation of AsA in blackcurrant fruit:

1. Mobilisation of AsA to fruit from storage pools.
2. Synthesis of AsA in photosynthetic tissues and transfer to fruit via phloem.
3. Synthesis of AsA in fruit from translocated sugars.

Headlines

- AsA accumulates in blackcurrant fruit during fruit expansion stages
- AsA accumulation is associated with high fruit biosynthetic capacity via the L-galactose pathway
- AsA accumulation is associated with low AsA turnover
- Translocation of AsA to fruit from either storage pools or from leaves does not significantly contribute to fruit AsA
- A model for fruit AsA accumulation is presented

AsA and carbohydrate accumulation in blackcurrant fruit

Fruit were harvested throughout the growing season and stored at -80^{o}C prior to analysis. AsA concentration increased significantly during the period of bud growth and differentiation to flowers but then showed a slow steady decline (Fig. 1.1A). At the very early stages of fruit development (closed flowers), AsA concentration on all three genotypes was similar, however the decline of AsA concentration was significantly greater in Hedda during fruit development than in other genotypes. Although AsA concentration declined during fruit development, there was a marked increase in total AsA per fruit during the early stages of development (until fruit started to develop red colouration) which then remained constant as the fruit developed towards maturity (Fig. 1.1B). These data are similar to those published previously (Viola *et al.*, 2000). Although 8982-6 had a higher AsA concentration than Baldwin, the total AsA per fruit was higher in Baldwin as a result of the higher average fruit weight.

In all genotypes there was an increase in sugar concentration throughout fruit development with the exception of 8982-6 in which a large spike in sugar concentration (mainly sucrose, data not shown) was associated with the open flower stage (Fig. 1.1C). In terms of total sugar accumulation, all three genotypes demonstrated a two phase accumulation of sugars with an initial accumulation occurring during developmental stages 1-3 followed by a leveling off and then a second phase of sugar accumulation occurring in stages 4-6 (Fig. 1.1D).

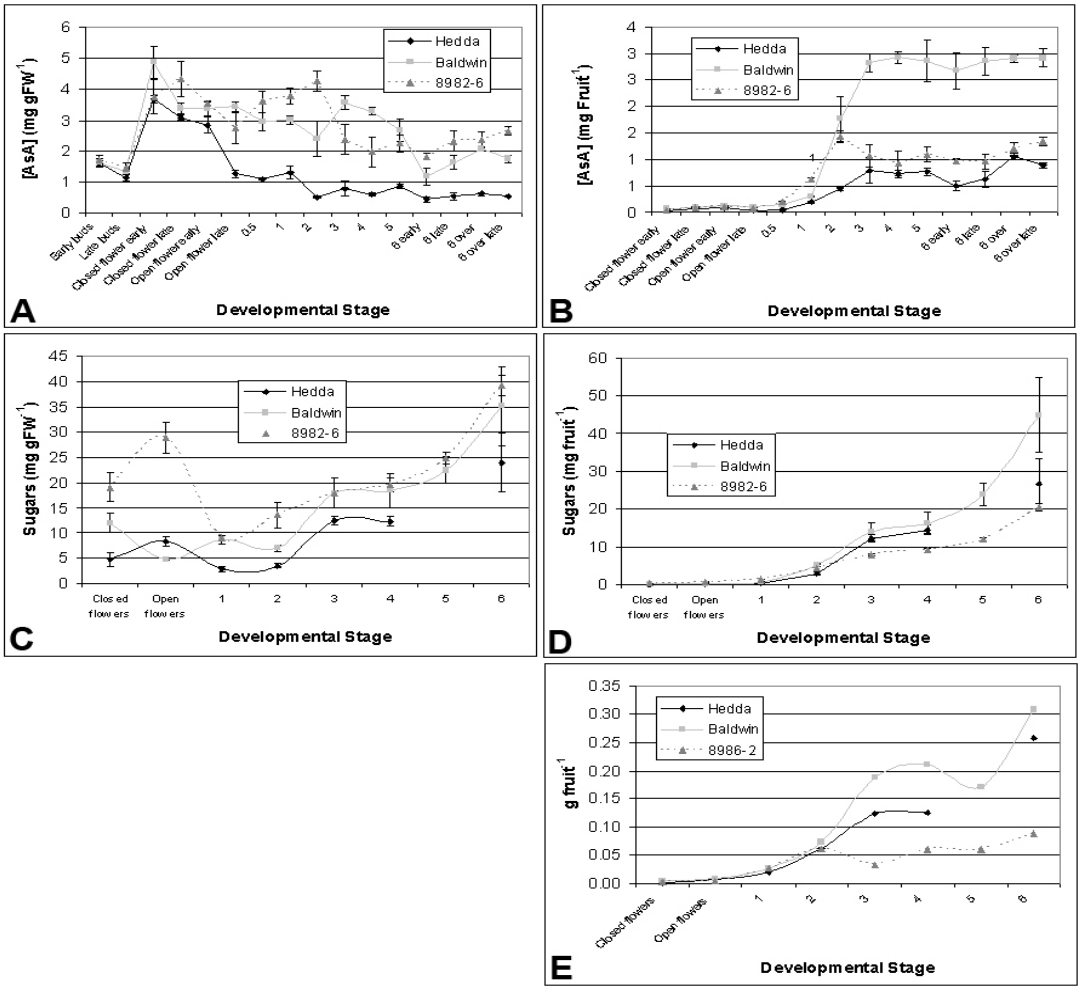


Figure 1.1 Accumulation of AsA, sugars and dry matter by blackcurrant fruit during development

Buds or fruit were collected from bushes over the course of development, snap frozen in liquid N_2 and stored at -80^{o}C until extraction and analysis as described in materials and methods. A – AsA concentration in fruit (mg gFW^{-1}); B – Total AsA per fruit (mg) based on the average mass of 50 fruit; C – Sugar concentration in fruit (mg gFW^{-1}); D – Total sugars per fruit (mg) based on the average mass of 50 fruit; E – Fruit dry weight (mg) based on the average of 50 fruit. Fruit developmental stages were 0.5, green < 4 mm diameter; 1 green 4-6 mm diameter; 2, green 8-12 mm diameter; 3, green-red; 4, red-green; 5, red; 6, black. Ripe fruit were harvested either as soon as they appeared to be ripe (6 early) or after 1, 2, or 3 weeks (6 late, 6 over and 6 over late respectively).

Accumulation of dry matter in cvs Hedda and Baldwin showed a similar pattern to that of sugars however, there was little increase in dry matter after stage 2 in genotype 8982-6 suggesting that the high AsA concentration observed in these fruit may be a result of limited fruit expansion.

Effect of fruit position on AsA accumulation

To further examine the accumulation of AsA, individual Baldwin fruit were extracted at different times during development to determine whether the position of fruit on the strig affected the accumulation of sugars and AsA. Fig. 1.2 shows the developmental stage of fruit taken for the experiment while Fig. 1.3 shows the AsA concentration of fruit at a particular strig position at different stages of development. Although there did appear to be a gradient of AsA concentration along the strig at certain stages (e.g. open flowers, 9 May) this pattern was not consistent throughout development.

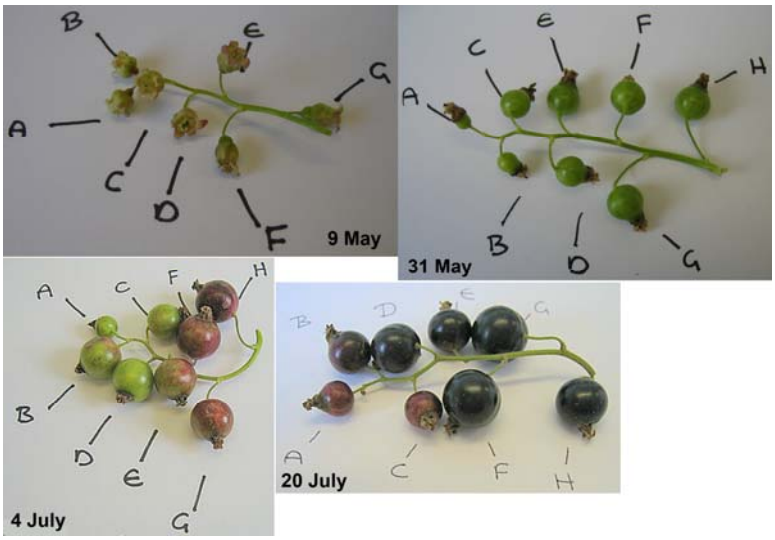


Figure 1.2 Baldwin fruit development at sampling dates to determine effect of strig location on fruit AsA content

Fruit were sampled on the dates shown. The letter designation for each fruit relates to that shown in Fig. 5.

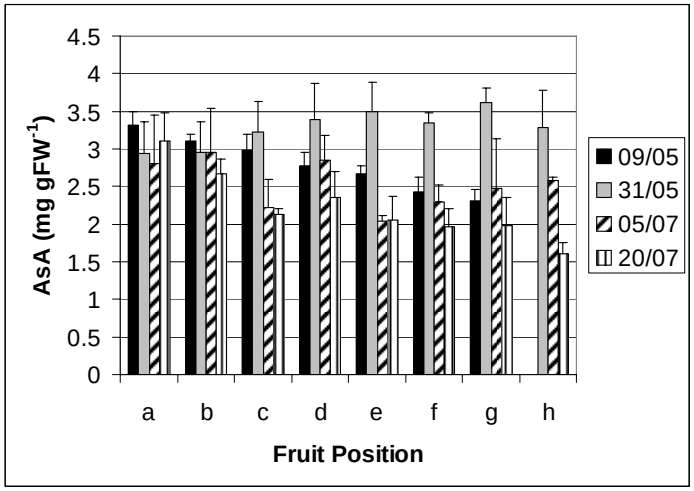


Figure 1.3 Effect of strig location AsA accumulation in Baldwin fruit

Fruit were sampled on the dates shown and individual fruit were extracted and AsA concentration estimated by HPLC. Three individual strigs were sampled and data shown are mean ± SE. For Fruit position see Fig. 4.

AsA and carbohydrate pools in blackcurrant tissues

To gain further insight into the accumulation of fruit AsA and carbohydrates, whole plants of cv. Baldwin were harvested throughout the annual cycle and AsA, sugar and starch concentrations in different tissues determined. Fruit represented the largest plant AsA reservoir with the ripe fruit of a plant standardised to 100 g DW stem containing 0.8 g AsA (Fig. 1.4A). This was considerably in excess of the AsA found in all other tissues combined which peaked at approximately 0.3 g. In addition to fruit, a second transient AsA pool was represented by photosynthetic tissues which contained approximately 0.15 g AsA throughout the summer months before declining as leaves began to senesce. The AsA pool peaked in stem tissues coincident with summer shoot growth and elongation prior to falling back as the new growth hardened. In the roots, AsA pools peaked during the winter months.

These data clearly demonstrate the absence of storage pools of AsA and eliminate the hypothesis of mobilisation of AsA from storage pools to fruit.

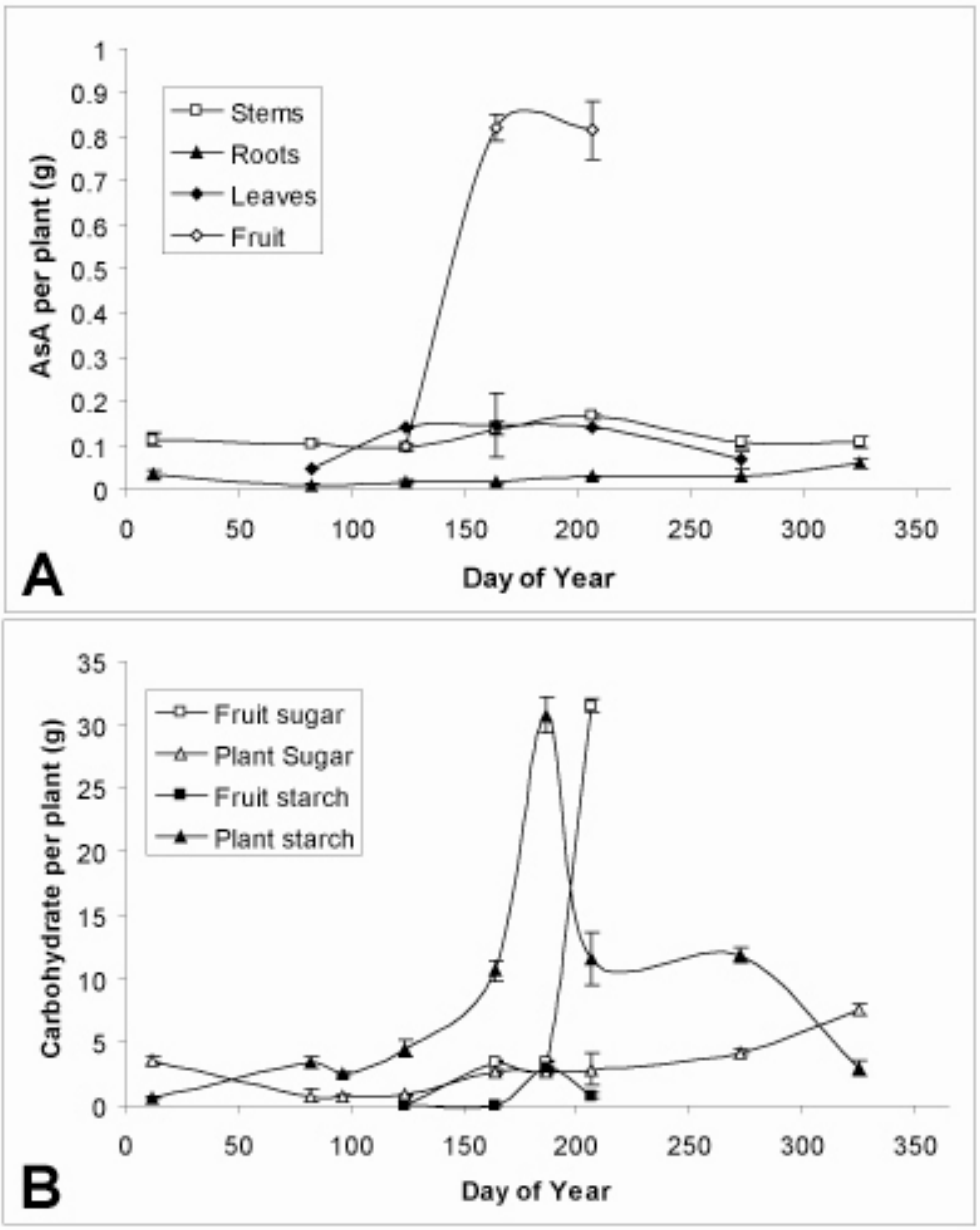


Figure 1.4 AsA and carbohydrate pools in cv. Baldwin plants over annual growth cycle

Plants were harvested at the times shown and fruit, leaves, stems and roots lyophilised and weighed. AsA was extracted and quantified by HPLC. Data are presented normalised to plants consisting of 100 g DW stem and represent mean values ± SE (n = 3). Panel A – Ascorbic acid, Panel B – Carbohydrate. For simplicity, values obtained from leaves, stems and roots were combined and expressed as plant sugar or plant starch.

Data presented in Fig. 1.4B illustrate large temporal fluctuations in non-fruit carbohydrate which contrasted with the data obtained for AsA (for simplicity root, leaf and stem values were combined). Early in the year (day 12; 12 January), plant starch reserves were almost completely depleted although the pool of soluble sugars was maintained at approximately

3.5 g in a plant with a 100 g DW stem. Between January and late March (~ day 80), starch levels recovered slightly at the expense of the sugar pool followed by a slight drop in starch levels associated with bud burst and early leaf development. Following leaf emergence (~ day 100) there was a large accumulation of starch which continued until the development of mid-ripe fruit (~ day 190). Starch accumulation was observed in roots, stems, leaves (data not shown) and fruit (Fig. 1.4B). Subsequently, starch levels in all plant tissues rapidly decreased and this was associated with a massive increase in soluble sugars in fruit. Assuming that all of the starch lost from other plant tissues was translocated to fruit, approximately two thirds of the sugars found in fruit at harvest resulted from starch mobilisation. Following fruit harvest, starch levels within the remainder of the plant remained stable until leaf fall (~ day 270). Following leaf fall, there was a strong decline in plant starch levels associated with a slight increase in soluble sugars, possibly associated with the acquisition of cold-tolerance as has been proposed in other perennial shrubs (Jones *et al.* 1999; Palonen 1999).

Translocation of AsA from leaves to fruit

Previous work has demonstrated the capacity for long-distance transport of AsA from source (leaf) to sink (non-photosynthetic) tissues in both model and crop plants (Franceschi and Tarlyn, 2002; Tedone *et al*., 2004) however, due to technical limitations serious attempts to determine the contribution of transport to sink AsA concentration has not previously been determined. In the current project several indirect methods were used to determine the contribution of AsA transport to fruit AsA concentration at maturity. The data suggest that transport made a minimal contribution to fruit AsA concentration at harvest.

i. Mechanism of phloem unloading in developing blackcurrant fruit

Following leaf labelling with the phloem mobile tracer CFDA, dye was observed within the phloem of the fruit pedicel demonstrating uptake of the dye by leaf phloem and transport to fruit (Fig. 1.5A). Similarly, dye was observed with phloem cells within the fruit mesophyll, however in this instance dye was also observed in a diffuse pattern in cells adjacent to the phloem (Fig. 1.5B).

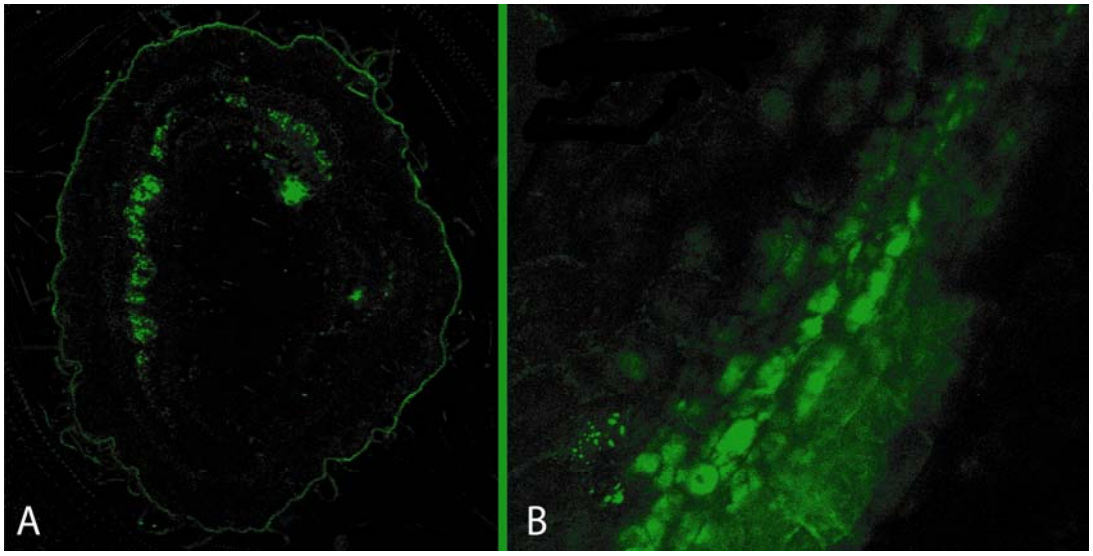


Figure 1.5 Fluorescent imaging of CFDA within blackcurrant tissues

CFDA was loaded into leaves of intact cv. Baldwin plants via leaf 'flaps' and allowed to translocate for 15 h. Hand sections (2 mm) of fruit pedicel were prepared with a razor blade and fruit were bisected by hand. Images were recorded using a Leica SP2 microscope equipped with an argon laser and tissue excitation was undertaken at 488 nm and emission signals recorded at 517-552 nm. Panel A – Cross section through a fruit pedicel with CF fluorescence restricted to phloem strands with little diffusion of dye to adjacent cells. Panel B – Cross section through a developing fruit where CFDA can be observed both in phloem and diffusing into adjacent cells.

Membrane CFDA is able to diffuse into phloem at the site of loading where endogenous esterases remove the acetate groups generating the membrane impermeable compound CF. As a result, the compound becomes trapped within the phloem and can only be unloaded via plasmodesmata therefore representing a marker for symplastic unloading. The data presented here demonstrate symplastic phloem unloading in blackcurrant fruit hence any compounds that can be taken up by source phloem in blackcurrant leaves will inevitably accumulate in fruit.

ii. Uptake of [1-^{14}C]AsA by blackcurrant leaves

In order to determine the capacity for the uptake of AsA by source leaf phloem, leaf discs were prepared, incubated with either [U-^{14}C] sucrose, [1-^{14}C] or L-[1-^{14}C]Gal and exposed to photographic film.

Data presented in Fig. 1.6 shows strong uptake and labelling of sucrose, the primary transport sugar, in blackcurrant leaf phloem. This data demonstrates the utility of the technique and is the specificity of uptake is demonstrated by the capacity of the sucrose transport inhibitor *p*-chloromercuribenzene sulphonic acid (PCMBS) to abolish the

labelling pattern. In contrast, uptake of [1-^{14}C] AsA is barely detectable although there is apparent weak uptake of the AsA precursor L-Gal. These data suggest that AsA transport is unlikely to contribute significantly to fruit AsA accumulation although the finding that AsA precursors are weakly accumulated in combination with the observation of symplastic phloem unloading in fruit suggests the possibility that transport of these compounds combined with their conversion to AsA in the fruit may represent a potential mechanism for AsA accumulation. On the other hand given the exceptionally low concentration of L-Gal found in leaf tissues (2 nmol g FW^{-1}; Gatzek *et al.*, 2002) this route is likely to be significant.

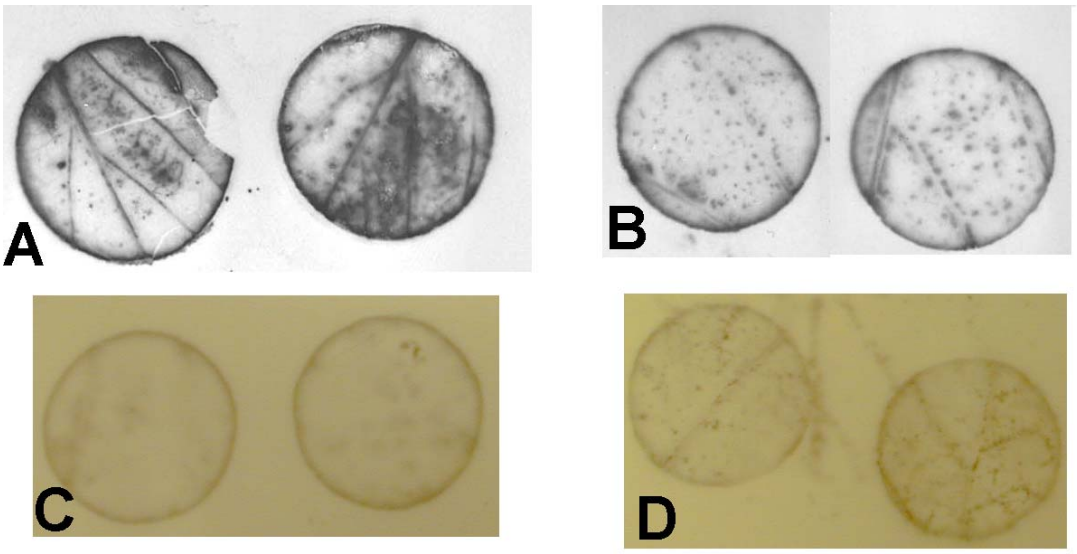


Figure 1.6 Uptake of ^{14}C labelled compounds by blackcurrant leaves

Leaf discs were prepared from source leaves of cv. Baldwin and incubated for 18h in the dark with the appropriate compound (final concentration 5 mM). Leaf discs were then washed 5 times in fresh buffer without label, lyophilised and exposed to photographic film for 7 days at -80^{o}C. Leaves were incubated with [U-^{14}C]sucrose (panel A), [U-^{14}C]sucrose + 2 mM PCMBS (panel B), [1-^{14}C]AsA (panel C) or L-[1-^{14}C]Gal (panel D) and images are representative of observed results in ten discs.

iii. Presence of AsA in phloem exudates from blackcurrant leaf petiole

Given the very low uptake of [1-^{14}C]AsA by source phloem in blackcurrant leaves, further experiments were undertaken to determine the presence of AsA in transport phloem. Leaves were removed from field-grown plants with petioles intact. The petioles were re-cut under water and transferred to buffer solutions consisting of either 15 mM EDTA pH 7.5 to sequester Ca^{2+} and hinder callose gelation or 5 mM $CaCl_2$ in order to promote callose gelation and prevent phloem exudation. Leaves were transferred to a saturated

atmosphere to exude for 90 min, exudates analysed by HPLC and AsA peak area of the chromatogram determined. The presence of a peak attributable to AsA was observed in all exudates analysed. Inclusion of EDTA in the exudation medium resulted in an enhanced AsA peak and the difference in the AsA peak areas between EDTA and $CaCl_2$ exudation media is interpreted as the contribution of AsA from the phloem (Fig. 1.7). These data therefore demonstrate that AsA is present in the phloem of blackcurrants. One major difficulty of the method is that there is no reliable way to measure the volume of phloem exuded therefore although the presence of AsA in the phloem has been demonstrated no data regarding the relative contribution of phloem transported to fruit AsA concentration at harvest is available.

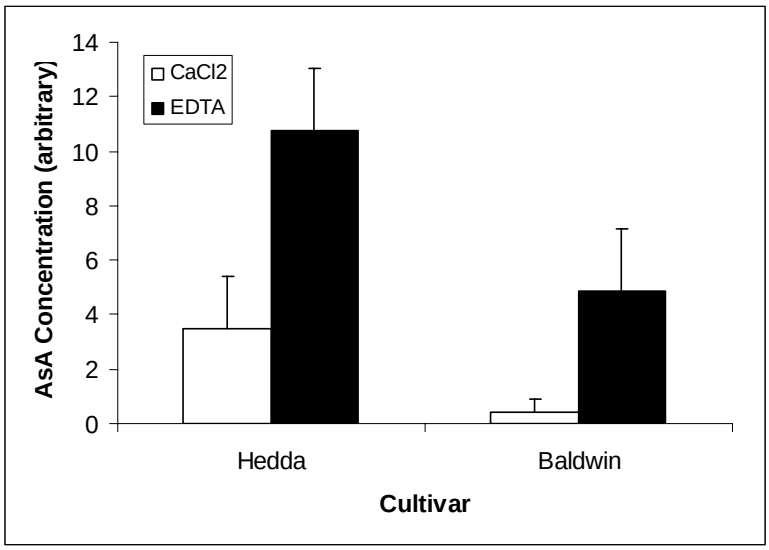


Figure 1.7 Presence of AsA in petiole exudates from blackcurrant leaves

Leaves were removed from plants and petiole exudates were collected into $CaCl_2$ (excluding phloem content) or EDTA (including phloem content). AsA concentration was estimated as chromatogram peak area following HPLC.

iv. Translocation of [1-^{14}C]AsA from leaves to fruit

AsA in the phloem may arise either from direct uptake of the compound or alternatively from the uptake of precursors and subsequent conversion to AsA within the phloem itself as was previously demonstrated in other crops (Hancock *et al.*, 2003). In order to determine whether AsA was directly taken up by source leaf phloem and translocated to fruit, [1-^{14}C]AsA was supplied to leaves via leaf 'flaps' and radioactivity in leaves and fruit quantified.

Initial experiments showed that radioactivity travelled from leaves to fruit on the same node but that transport between nodes was negligible (Fig. 1.8), therefore only translocation between leaves and fruit of the same node was quantified.

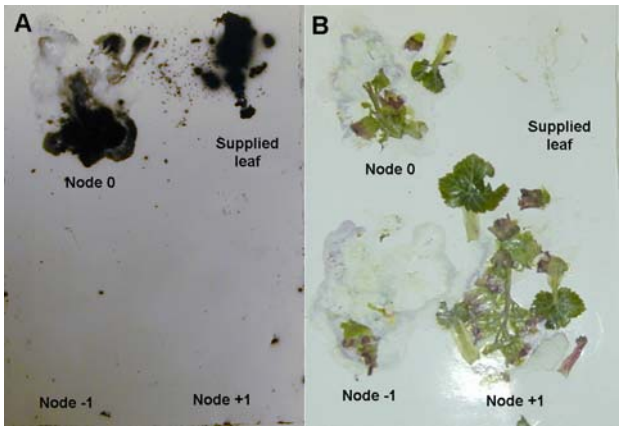


Figure 1.8 Transfer of [1-^{14}C]AsA from leaves to flowers in blackcurrant cv. Baldwin

Leaves were supplied with [1-^{14}C]AsA via 'flaps' on intact plants and allowed to translocate for 15 h. Leaves and flowers were removed from the treated node (node 0), the node above (node +1) and the node below (node -1), pressed between card, lyophilised and exposed to X-ray film. Panel A, distribution of radioactivity as determined by exposure of X-ray film; panel B, photograph of tissue pressed onto card.

In a subsequent experiment, all of the leaves on a single node of cv. Baldwin plants bearing developing green fruits were supplied with [1-^{14}C]AsA via 'flaps' for 48 h (Fig. 1.9). Following incubation, both leaves and fruit were removed and individually extracted to determine the activity and distribution of radioactivity. [1-^{14}C]AsA was strongly taken up by leaves over the course of the incubation with between 3.2 x 10^6 and 9.5 x 10^6 DPM gFW^{-1} tissue absorbed (Fig. 1.9). On the contrary, the quantity of label translocated to fruit was significantly lower with some fruit taking up very little radioactivity (< 100 DPM gFW^{-1}) and while the majority of fruit contained approximately 10^4 DPM gFW^{-1} with only one fruit absorbing significantly more radioactivity (5.2 x 10^4 DPM gFW^{-1}). These data again suggest very little translocation of AsA from leaves to fruit with fruit containing only approximately 1% of the levels of radioactivity observed in leaves. Analysis of the distribution of radioactivity in fruit and leaves revealed significant breakdown of AsA with only approximately 50% of activity in leaves remaining as AsA while the proportion in fruit was even lower at approximately 30% (Fig. 1.9).

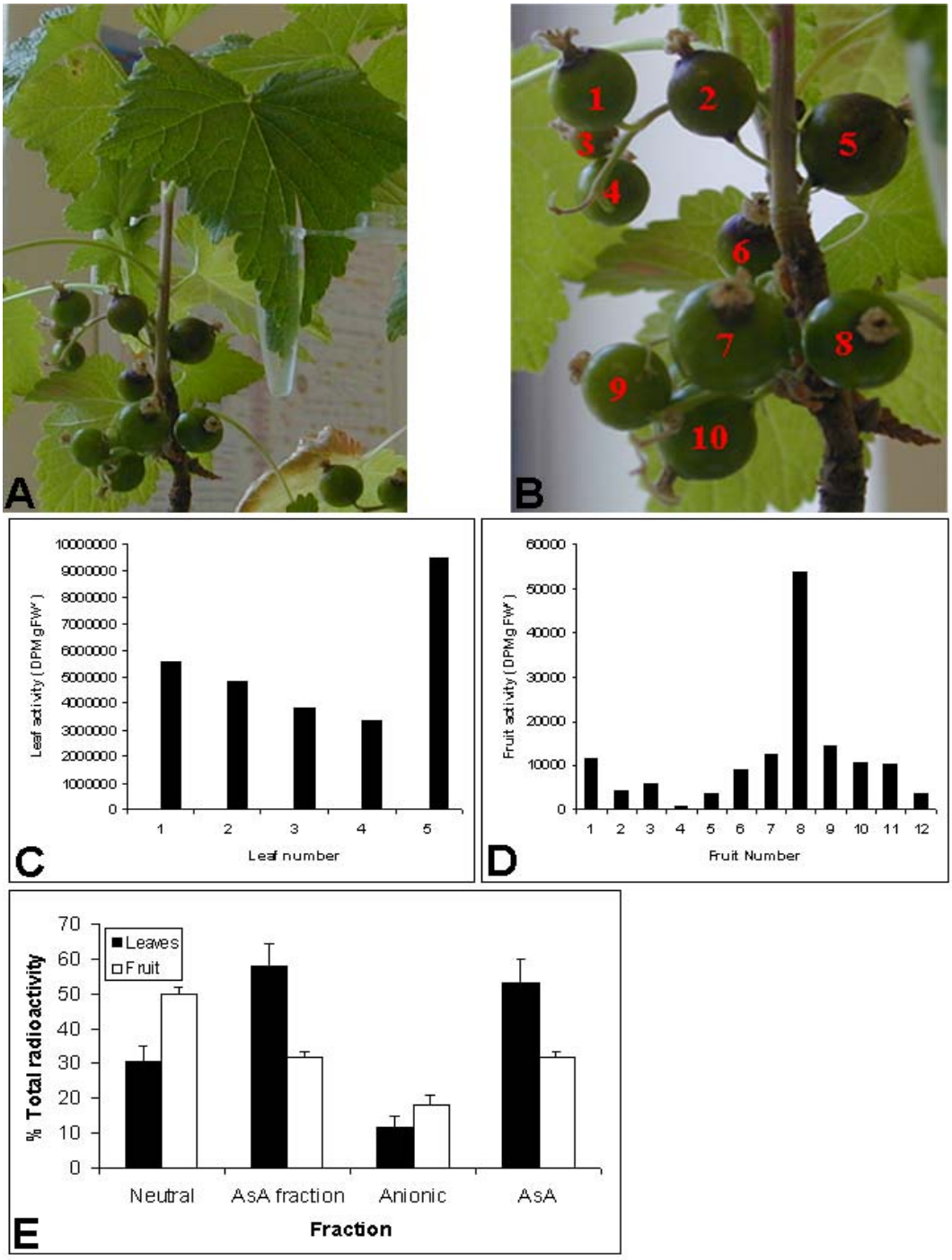


Figure 1.9 Translocation of [1-^{14}C]AsA from leaves to fruit in intact blackcurrant plants

Leaves were supplied with [1-^{14}C]AsA via 'flaps' for a period of 48 h (panel A) after which leaves and individually numbered fruit were removed and extracted (panel B, fruit 11 and 12 hidden). Total radioactivity in both leaves (panel C) and fruit (panel D) was determined by liquid scintillation counting and the distribution of radioactivity in each tissue was determined by a combination of SAX SPE and HPLC (panel E).

v. Translocation of unlabelled AsA and precursors from leaves to fruit

To determine whether enhancing leaf AsA levels may increase translocation of the compound from leaves to fruit, leaves were incubated with 50 mM AsA or 50 mM L-GalL (the immediate precursor of AsA) for 48 h and the impact on fruit AsA concentration was determined. Incubation of leaves with L-GalL resulted in a two-fold increase in leaf AsA concentration while incubation with AsA was even more effective raising the concentration in leaves almost 3-fold (Fig. 1.10). Despite the impact of incubation of leaves with AsA, no difference in fruit AsA levels were observed. On the contrary, incubation of leaves with L-GalL resulted in a significant increase in fruit AsA concentration.

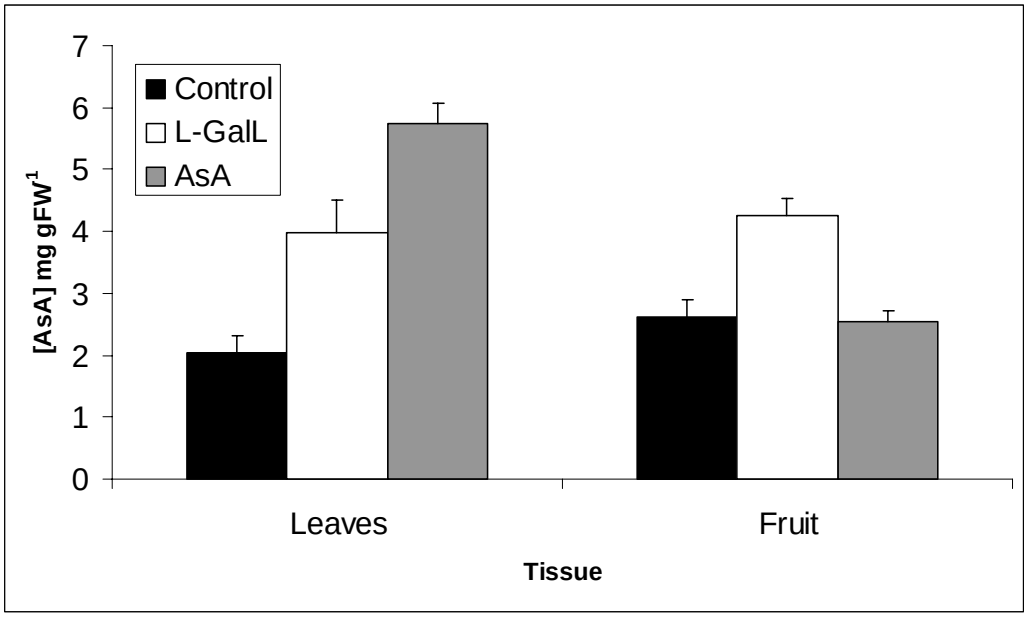


Figure 1.10 Impact of incubation of blackcurrant leaves with AsA or precursors on fruit and leaf AsA concentration

Leaves on intact cv. Baldwin plants were incubated with 50 mM AsA or L-GalL via 'flaps' for 48h. Immediately following incubation leaves and fruit of the same node were removed and AsA extracted and quantified by HPLC. Data are presented as mean ± SE of three independent experiments.

The data again suggests limited transport of AsA from leaves to developing fruit. The increase in fruit AsA concentration following incubation with L-GalL again suggests that precursors may be transported from leaves to fruit thereby contributing to fruit AsA accumulation however, like L-Gal cytoplasmic concentrations of L-GalL would be extremely low under normal conditions. It seems likely that under the conditions of the

experiment where the cytoplasmic L-GalL concentration was massively raised the compound may travel passively from leaves to fruit (Fig. 1.11). In the leaf mesophyll which has a relatively low pH of approximately 6.5, L-galactonic acid (L-GalA) would be in the lactone ring form (L-GalL) and therefore uncharged. The result would be that the compound would have a fair degree of membrane solubility and could passively diffuse into the phloem sieve tube. In this cellular compartment the higher pH (~ 8.0) would favour delactonisation to L-GalA and the compound would acquire a negative charge effectively trapping it within the phloem sieve element. As the data presented above demonstrates symplastic phloem unloading into blackcurrant fruit, L-GalA would be unloaded into the fruit mesocarp where the lower pH would promote relactonisation and subsequent to AsA.

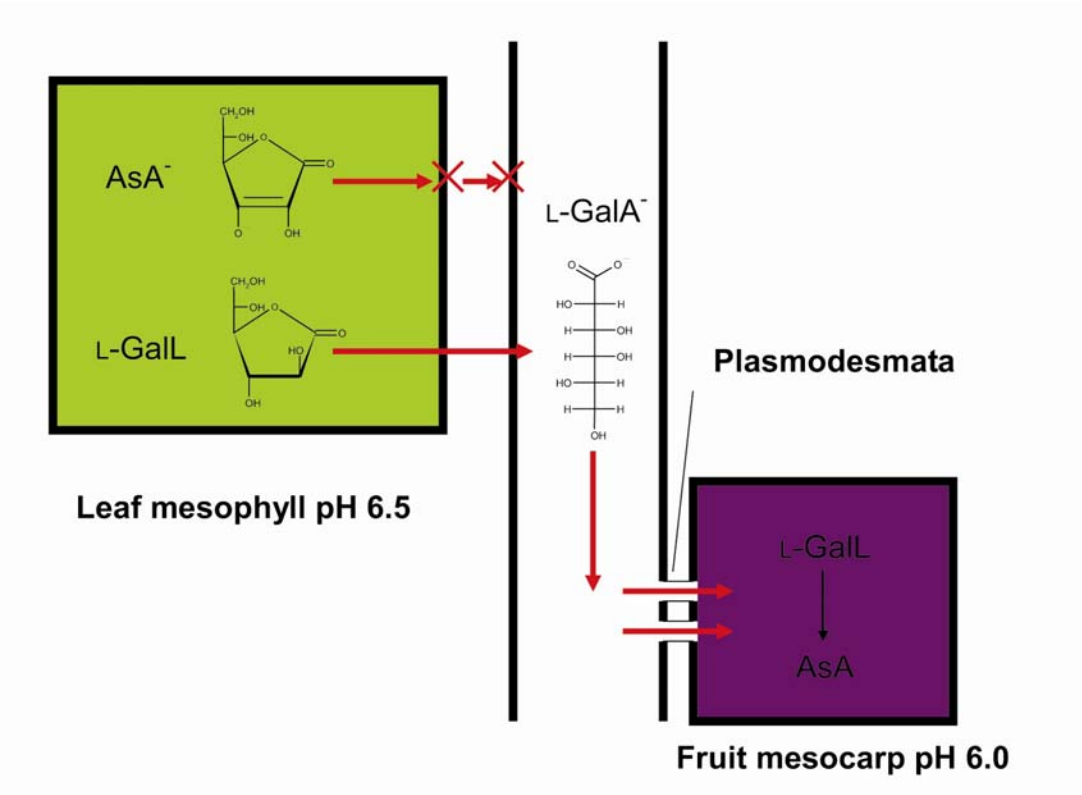


Figure 1.11 Mechanism of passive translocation of L-GalL from leaves to fruit in blackcurrant

In its uncharged lactone form L-GalL can passively diffuse into the phloem sieve element and be translocated to fruit unlike the charged AsA ion. For a full explanation refer to text.

vi. Leaf AsA biosynthetic capacity

Assuming translocation of AsA from leaves to fruit were a significant source of fruit AsA, an increased leaf AsA demand might be expected at the time of maximal fruit AsA accumulation. This is turn might be expected to result in enhanced leaf AsA biosynthetic capacity at the time of maximum fruit demand therefore, studies were undertaken to determine how leaf biosynthetic varied on plants bearing fruit at different stages of development. In initial experiments, leaf discs were incubated with precursors of different AsA biosynthetic pathways to determine their impact on AsA concentration. Incubation with precursors of the L-galactose pathway (L-Gal and L-GalL; Fig. 1) significantly enhanced leaf AsA concentration in the genotypes Hedda, Baldwin and 8982-6 at most stages of plant development (Fig. 1.12). In addition, precursors of the L-gulose pathway (L-Gul and L-GulL; Fig. 1) enhanced leaf AsA concentration at certain stages of plant development in the genotypes Hedda and 8982-6 only (Fig. 1.12). Precursors of the D-galacturonic acid and *myo*-inositol pathways (Fig. 1) were ineffective at increasing leaf AsA concentration in all genotypes at all stages of development.

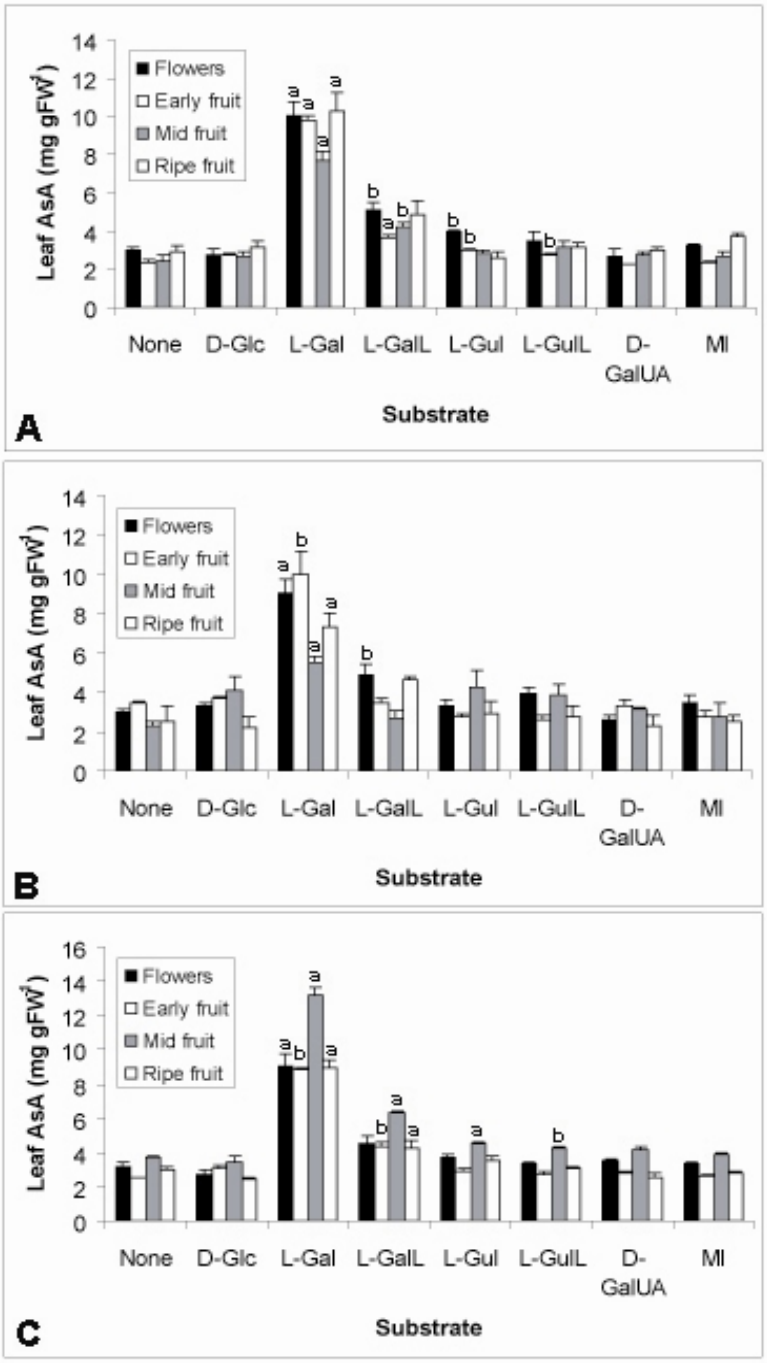


Figure 1.12 Impact of incubation with AsA precursors on leaf AsA levels in three blackcurrant genotypes

5 mm leaf discs were prepared from plants bearing fruit of the appropriate stages and incubated in the dark with the appropriate precursor (25 mM) for 18 h. Leaf discs were then extracted and AsA quantified by HPLC. Data are presented as mean ± SE of three independent experiments and values significantly different from those of leaves incubated in the absence of substrate are indicated (a, $P < 0.01$; b, $P < 0.05$). Panel A, Hedda; Panel B, Baldwin; Panel C, 8982-6.

In order to undertake a more detailed analysis of leaf AsA biosynthetic capacity at different stages of development, leaf discs were supplied with D-[U-^{14}C]Man (an early precursor of both the L-galactose and L-gulose pathways) and the proportion of radioactivity incorporated into [^{14}C]AsA determined. In the three genotypes examined, leaf biosynthetic capacity was high in plants that bore closed or open flowers but fell significantly as plants matured such that leaf biosynthetic capacity in plants that bore green fruit was approximately half of that in plants that bore flowers (Fig. 1.13). Thus leaf biosynthetic capacity fell at the time of greatest fruit AsA demand. These data provide further evidence to suggest that translocation of AsA from leaves to fruit is unlikely to provide a significant contribution to fruit AsA accumulation.

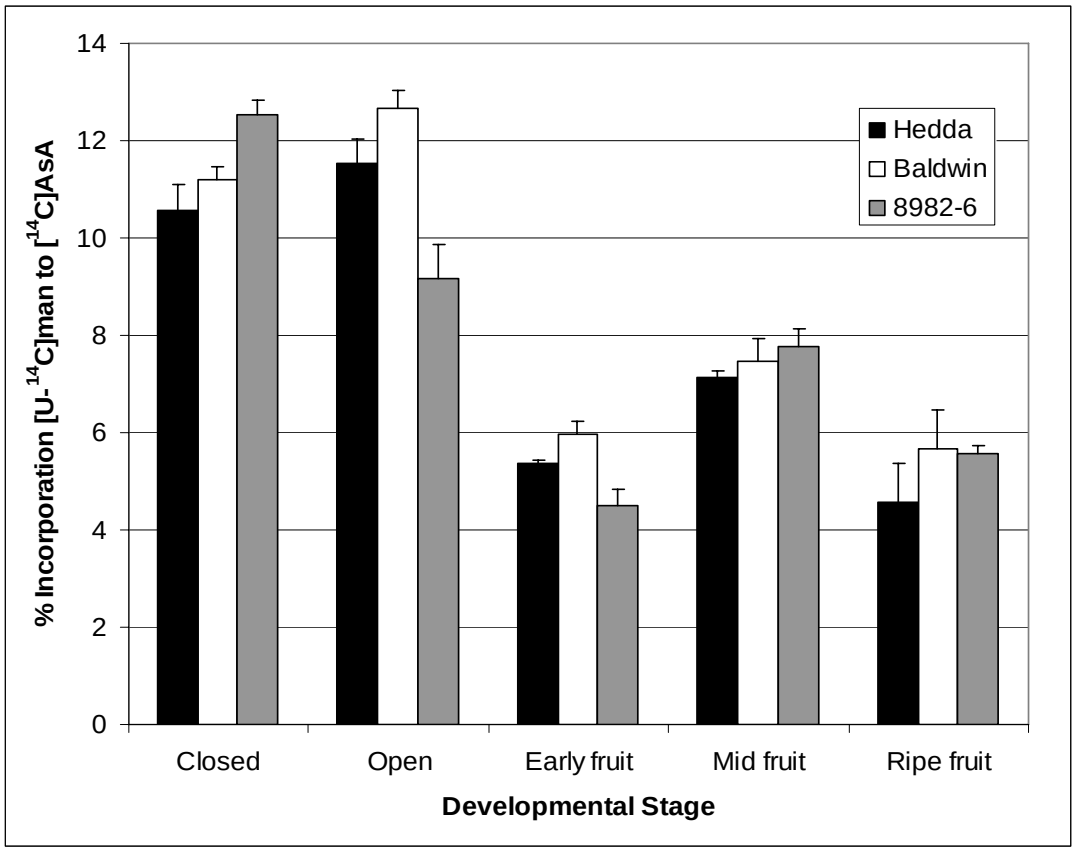


Figure 1.13 Incorporation of D-[U-^{14}C]Man to [^{14}C]AsA in leaves of blackcurrant plants bearing fruit at different stages of maturity

5 mm leaf discs were prepared from plants bearing fruit at the appropriate stage of maturity and incubated with D-[U-^{14}C]Man for 4 h. [^{14}C]AsA was extracted and quantified as described in the text and values are expressed as the % of metabolised label incorporated. Values represent mean ± SE of three independent experiments

Synthesis and metabolism of AsA by blackcurrant fruit

i. Supply of potential AsA precursors to fruit

Fig. 1.14 shows the effect of incubation with 25 mM precursor for 18 h on the ascorbate concentration of fruit at different stages of development. In all genotypes, precursor supply had the greatest effect on AsA concentration at the early developmental stages (flowers and early fruit). In these stages both L-Gal and L-GalL enhanced fruit AsA content compared with control fruit (buffer only), strongly suggesting the operation of the L-galactose pathway. In genotype 8982-6, L-GulL also had a statistically significant effect on fruit AsA content although this was much lower than that of L-GalL. Incubation of more developed fruit with L-Gal or L-GalL did not generally result in enhanced fruit AsA concentration compared with control treatment suggesting reduced AsA biosynthetic capacity during fruit maturation. In both Baldwin and 8982-6, incubation of ripe fruit with *myo*-inositol resulted in a statistically significant increase in AsA concentration compared with control.

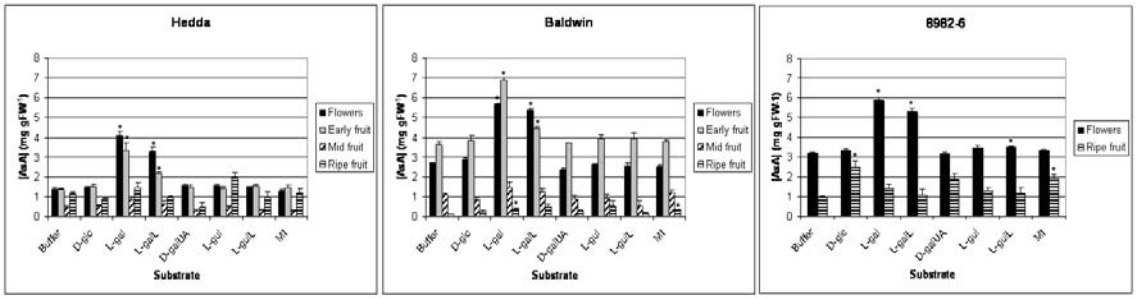


Figure 1.14 The effect of incubation with potential AsA precursors on blackcurrant fruit AsA concentration

Fruit were harvested from plants, bisected and incubated in 50 mM MES pH 6.5, 300 mM mannitol or in buffer with 25 mM of the appropriate precursor. After 18 h incubation, tissue was extracted and AsA concentration determined. AsA concentrations significantly different ($p<0.05$) from those in control fruit are marked with an asterisk.

ii. Determination of fruit biosynthetic capacity from D-[U-^{14}C]Man

Similar to the situation encountered in blackcurrant leaves, the data from precursor feeding studies suggested the dominant pathway for AsA synthesis in blackcurrant fruit was the L-galactose pathway with potential contributions from the L-gulose pathway. Fruit were

therefore incubated with D-[U-^{14}C]Man to analyse AsA biosynthetic capacity in greater detail. In corroboration with the data obtained from precursor feeding studies, flowers and early fruit demonstrated a high capacity for AsA synthesis in all genotypes (Fig. 1.15). As fruit ripened further, there was a sharp decline in AsA biosynthetic capacity with less that 1% of metabolised radioactivity incorporated into [^{14}C]AsA of fruit of maturity stage 3 or greater in all genotypes.

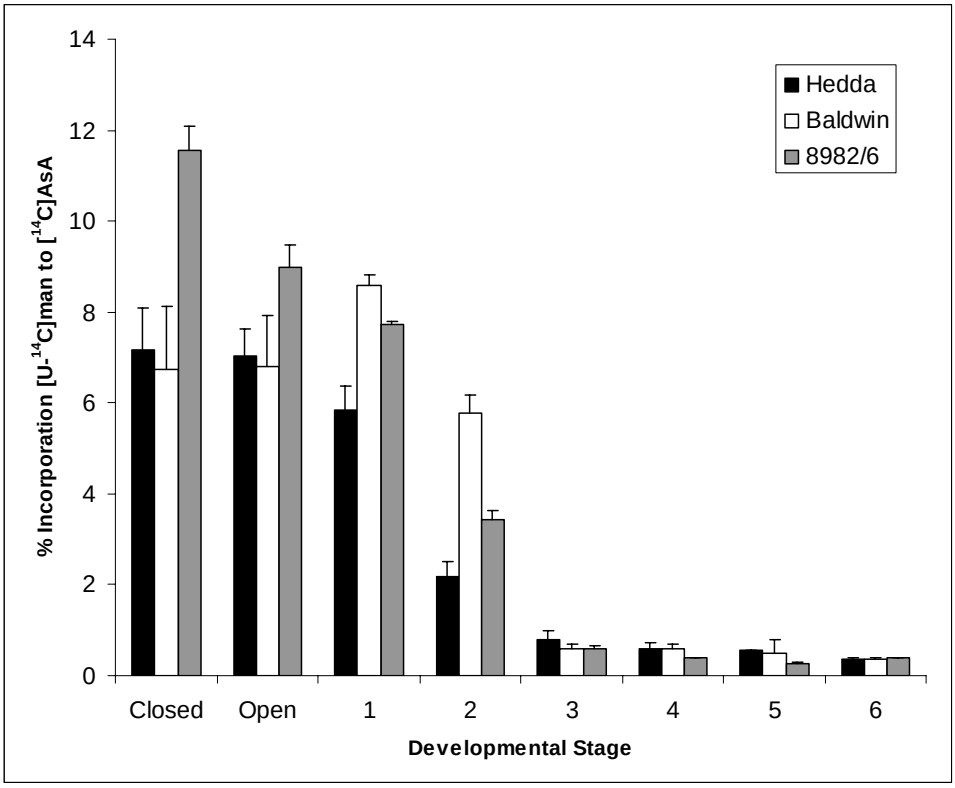


Figure 1.15 Incorporation of D-[U-^{14}C]Man into [^{14}C]AsA in fruit of three blackcurrant genotypes

Fruit were prepared by bisection and incubated with D-[U-^{14}C]Man for 4 h as described in the text. [^{14}C]AsA was extracted and quantified by HPLC with flow scintillation analysis as described in the text. Data are presented as mean incorporation of metabolized label into [^{14}C]AsA ± SE (n = 3).

The data correlate with fruit AsA accumulation, with high capacity associated with the early, AsA accumulating fruit stages (Fig. 1.15) and decreased biosynthetic capacity from stage 3 until fruit maturity associated with AsA maintenance. This data strongly suggest that AsA accumulation in blackcurrant fruit occurs by *in situ* synthesis via the L-galactose (L-gulose) pathway.

iii. Biosynthetic enzyme activities in fruit

In order to (i) determine which enzyme activities were contributing to reduced AsA synthesis as fruit matured and (ii) provide further information regarding operative pathways within fruit, attempts were made to extract and quantify enzyme activities from several of the potential biosynthetic pathways.

Initial attempts to extract enzyme activities using buffers that had previously been successful with other fruit tissues (Hancock *et al.*, 2003) failed. Several experiments were undertaken to optimize the extraction buffer and it was found that the addition of 5% (w/v) PVPP, 10 mg ml^{-1} gelatin and 10^4 dilution of *Aspergillus acetuleatus* pectinase (Sigma catalogue number P2611) gave the best enzyme activity recoveries.

Using the improved extraction buffer, a number of enzyme activities associated with AsA biosynthesis were quantified in fruit of three blackcurrant genotypes throughout development. We were able to detect and quantify a number of enzymes involved in the L-galactose pathway (Table 1.1) and were additionally able to record GDP-mannose-3,5-epimerase activity although accurate quantification was not possible. Of the enzyme activities quantified, no correlations could be determined between enzyme activity and biosynthetic capacity (Table 1.1). One possible reason for this observation is that none of the enzymes quantified are likely to catalyse rate limiting steps in AsA biosynthesis (Hancock and Viola, 2005). An alternative explanation is that despite the improvements in enzyme extraction methodologies outlined above, the extraction methods used were still not quantitative. In support of this hypothesis, previous detailed work on strawberry fruit has shown that even after method optimization, enzyme extraction from different stages of development resulted in variable recoveries of both total protein and individual enzyme activities (Souleyre *et al.*, 2004).

In addition to L-galactose pathway enzymes, we were able to detect galacturonic acid reductase activity in stage 4 fruit and ripe fruit of 8982-6. These data could suggest that there may be some contribution from this pathway to the maintenance of AsA levels in ripening fruit, however as L-galactono-1,4-lactone the immediate product of this reaction was a poor AsA precursor in stage 4 fruit (Fig. 1.14) and AsA accumulation had ceased by this developmental stage (Fig. 1.1), significant contribution is unlikely.

Although *myo*-inositol was shown to positively affect AsA concentration in ripe fruit of 8982-6, we were unable to detect *myo*-inositol oxygenase activity in any of the genotypes tested at any stage of fruit development (data not shown).

Genotype	Stage	Enzyme Activity (nmol min^{-1} g fw^{-1})				
		HK	**PGI**	**PMI**	**L-GalDH**	**GalUAR**
Hedda	2	8.79 ± 1.16	416 ± 26	ND	0.62 ± 0.05	ND
	4	0.97 ± 0.11	9.41 ± 2.22	0.74 ± 0.39	0.92 ± 0.06	20.1 ± 1.8
	6	1.00 ± 0.11	22.0 ± 1.7	ND	1.73 ± 0.23	ND
Baldwin	2	8.94 ± 0.36	534 ± 22	ND	0.78 ± 0.11	ND
	4	0.99 ± 0.21	13.2 ± 3.5	1.13 ± 0.07	2.64 ± 0.65	21.6 ± 2.0
	6	3.06 ± 0.78	18.9 ± 0.5	8.91 ± 1.80	1.81 ± 0.70	ND
8982-6	2	1.34 ± 0.08	29.5 ± 0.6	ND	2.91 ± 0.66	ND
	4	0.65 ± 0.09	15.0 ± 1.2	1.36 ± 0.79	1.33 ± 0.22	20.6 ± 1.2
	6	0.29 ± 0.07	24.1 ± 3.1	0.57 ± 0.08	1.63 ± 0.23	4.43 ± 1.44

Table 1.1 **R**ecovery of enzyme activities from blackcurrant fruit

Fruit were extracted in triplicate in 50 mM HEPES pH 8.0, 5% PVPP, 10 mg ml^{-1} gelatin, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1mM EGTA, 1mM benzamidine HCl, 0.5 mM PMSF and 10^{-4} dilution of pectinase. Enzyme activities were determined according to Hancock et al. *(2003) with the exception of GalUAR which was determined according to Agius* et al. *(2003).*

iv. Fruit AsA turnover

AsA is not a stable metabolic product and is subject to oxidation, recycling and further metabolism to other organic acids (Hancock and Viola, 2005) therefore AsA turnover may also play a significant role in control of fruit AsA concentration. For this reason, AsA turnover was determined following fruit loading with L-[1-^{14}C]AsA. In flowers and early stage fruit, AsA turnover was limited representing approximately 1% of the total AsA pool per hour however in more developed fruit, AsA turnover significantly increased representing approximately 3% of the total AsA pool h^{-1} (Table 1.2). In conjunction with the fruit labeling studies from D-[U-^{14}C]Man these data indicate that fruit AsA accumulation is associated with high biosynthetic capacity and low AsA turnover and that cessation of accumulation is associated with low biosynthetic capacity and high AsA turnover.

	Ascorbic Acid Turnover (% Total Pool h^{-1})		
	Hedda	Baldwin	8982-6
Flowers	1.09 ± 0.11^{a}	0.82 ± 0.09^{a}	0.97 ± 0.12^{a}
Early fruit	0.45 ± 0.03^{b}	1.41 ± 0.15^{a}	1.13 ± 0.05^{a}
Mid fruit	3.74 ± 0.45^{c}	3.22 ± 0.15^{b}	3.56 ± 0.42^{b}
Ripe fruit	3.90 ± 0.28^{c}	3.78 ± 0.54^{b}	3.78 ± 0.52^{b}

Table 1.2 Fruit AsA turnover in three blackcurrant genotypes at different stages of fruit ripening

Fruit were collected at the appropriate ripening stage, prepared by dissection and incubated with [1-^{14}C]AsA for 2 h as described in the text. Following incubation, one batch of fruit was extracted immediately and a further batch incubated for a further 24 h in the absence of label. AsA turnover was calculated from the specific activities of fruit AsA between samples processed immediately following labeling and those processed after 24 h incubation in the absence of label.

v. Impact of L-GalL supplementation

Experiments designed to investigate the potential role of leaf to fruit transport had shown that supplementation of leaves with L-GalL resulted in enhanced fruit AsA concentration. In order to determine the impact of such supplementation in the long term, leaves were supplied with 50 mM L-GalL for 48 h after which the substrate was removed and AsA concentration in both leaves and fruit was determined over a period of weeks post-treatment. As expected, both leaf and fruit AsA concentrations were significantly enhanced immediately post-treatment (Fig. 1.16). In leaves, AsA concentrations remained significantly higher in treated compared with control plants after 1 week however, there were no significant differences between fruit AsA concentrations in treated and control plants. After 2 weeks, there were no differences in AsA concentrations between treated and control plants in either leaves or fruit. These data indicate that under particular environmental conditions there was a defined fruit AsA concentration that the plant was able to control through a variety of metabolic and developmental responses.

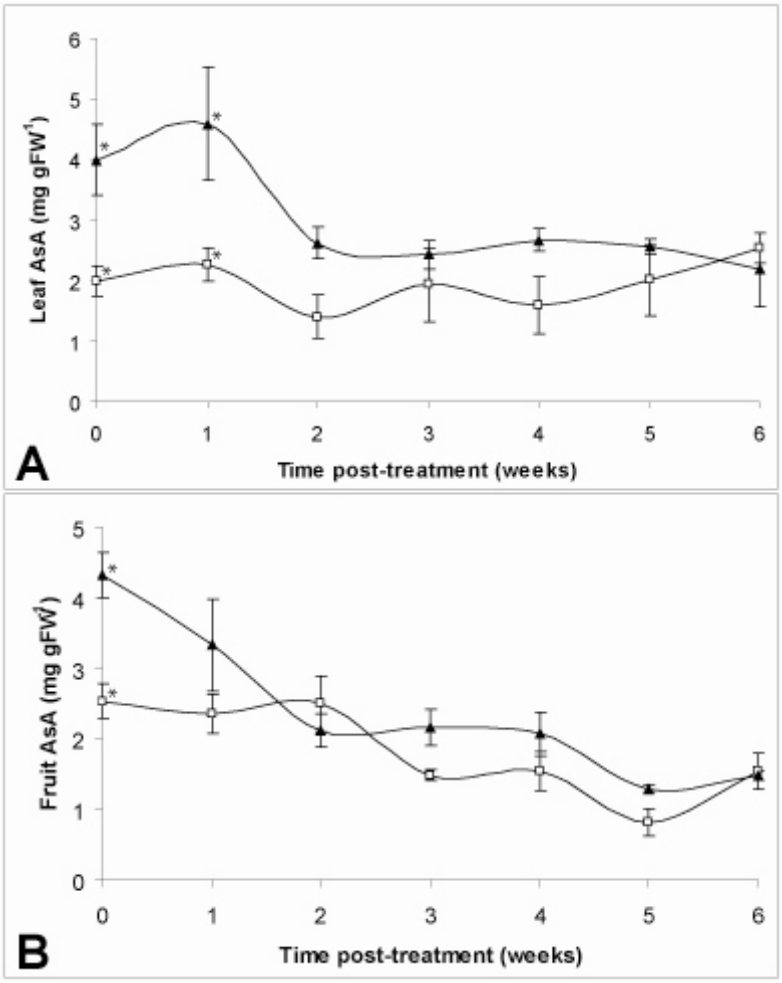


Figure 1.16 Effect of removal of L-GalL on leaf and fruit AsA concentration

Leaves were supplied with 50 mM L-GalL via 'flaps' for 48 h after which the substrate was removed. Leaves and fruit were sampled immediately after removal and the weekly for the subsequent six weeks. AsA was extracted from the tissues and quantified by HPLC as described in the text. Data is represented as mean ± SE of three independent experiments and values significantly different between treatments ($P < 0.05$) are indicated by asterisks. Panel A, leaf AsA concentration; Panel B, fruit AsA concentration.

vi. Impact of defoliation on fruit AsA accumulation

As sugars are the ultimate substrate for AsA biosynthesis, experiments were undertaken to determine the impact of substrate limitation on fruit AsA concentration. A series of plants were defoliated immediately following harvest in August 2005 and then left to develop normally in 2006. It was hypothesised that early defoliation would result in decreased starch retention over winter and therefore decreased substrate availability during the early

part of fruit development in the following year (Fig. 1.4). Fruit were harvested at ripeness in 2006 and several yield and quality parameters determined. The only fruit quality parameters to be significantly affected by the defoliation treatment were yield which was reduced by more than half and fruit water content which increased by about 15% (Table 1.3) possibly driven by the slight (although statistically insignificant) increase in sugar concentration. There were no changes in the fruit content of sugars, starch or AsA.

Quality Parameter	Undefoliated plants	Defoliated plants
Yield (g)*	1914 ± 115	876 ± 208
Water content (%)*	60.0 ± 1.3	69.6 ± 2.2
Glucose (mg gFW^{-1})	26.1 ± 1.4	29.2 ± 0.7
Fructose (mg gFW^{-1})	33.7 ± 1.6	36.0 ± 0.2
Sucrose (mg gFW^{-1})	13.5 ± 0.6	14.9 ± 0.4
Soluble sugars (mg gFW^{-1})	73.3 ± 3.4	80.2 ± 0.9
Starch (mg gFW^{-1})	0.26 ± 0.01	0.24 ± 0.02
Ascorbic acid (mg gFW^{-1})	2.91 ± 0.13	2.72 ± 0.09

Table 1.3 Impact of post-harvest defoliation on fruit quality parameters in fruit harvested in the following year

*Plants of cv. Baldwin were defoliated following harvest in 2005 as described in materials and methods. Following normal development, fruit were harvested at ripeness in 2006 and analyses undertaken to determine various quality parameters. Data are represented as mean ± standard error (n = 3). * Represents significant differences ($p < 0.05$) between values obtained from control and defoliated plants according as determined using the Students t-test.*

These data provide clear evidence that postharvest photosynthesis plays a significant role in the development of the following years' crop. However, the primary response of the plant to resource limitation is to reduce fruit yield while maintaining fruit composition. These data suggest that an abundance of resources might result in an increase of crop yield but would be unlikely to specifically enhance the fruit AsA concentration.

A model for AsA accumulation in blackcurrant fruit

Based on the data described above the following model is proposed for AsA accumulation in blackcurrant fruit. Immediately following bud burst, hydrolysis of stored starch provides structural carbohydrate to drive early leaf expansion (Fig. 1.17A). After initial leaf expansion, sugars are provided by photosynthesis and diverted to fruit where an active L-galactose pathway drives AsA accumulation. Limited AsA turnover also helps to drive accumulation. At this time significant quantity of photosynthate is allocated to starch synthesis in storage organs and a large starch pool is accumulated (Fig. 1.17B). As fruit

approach ripening, starch pools are hydrolysed and the released sugars transported to fruit. Sugars also continue to be provided by photosynthesis. Despite the large availability of substrate within the fruit, AsA biosynthesis is strongly down regulated while turnover is up regulated resulting in a cessation of AsA accumulation (Fig. 1.17C). Up to two thirds of the sugars found in ripe fruit are supplied from the mobilisation of starch stores. Following harvest, declining photosynthetic capacity is sufficient to cover maintenance and respiration and starch stores remain undepleted (Fig. 1.17D) until leaf fall when starch is mobilised to provide sugars for maintenance and respiration and to increase the cellular osmotic potential and provide tissue freezing tolerance (Fig. 1.17E).

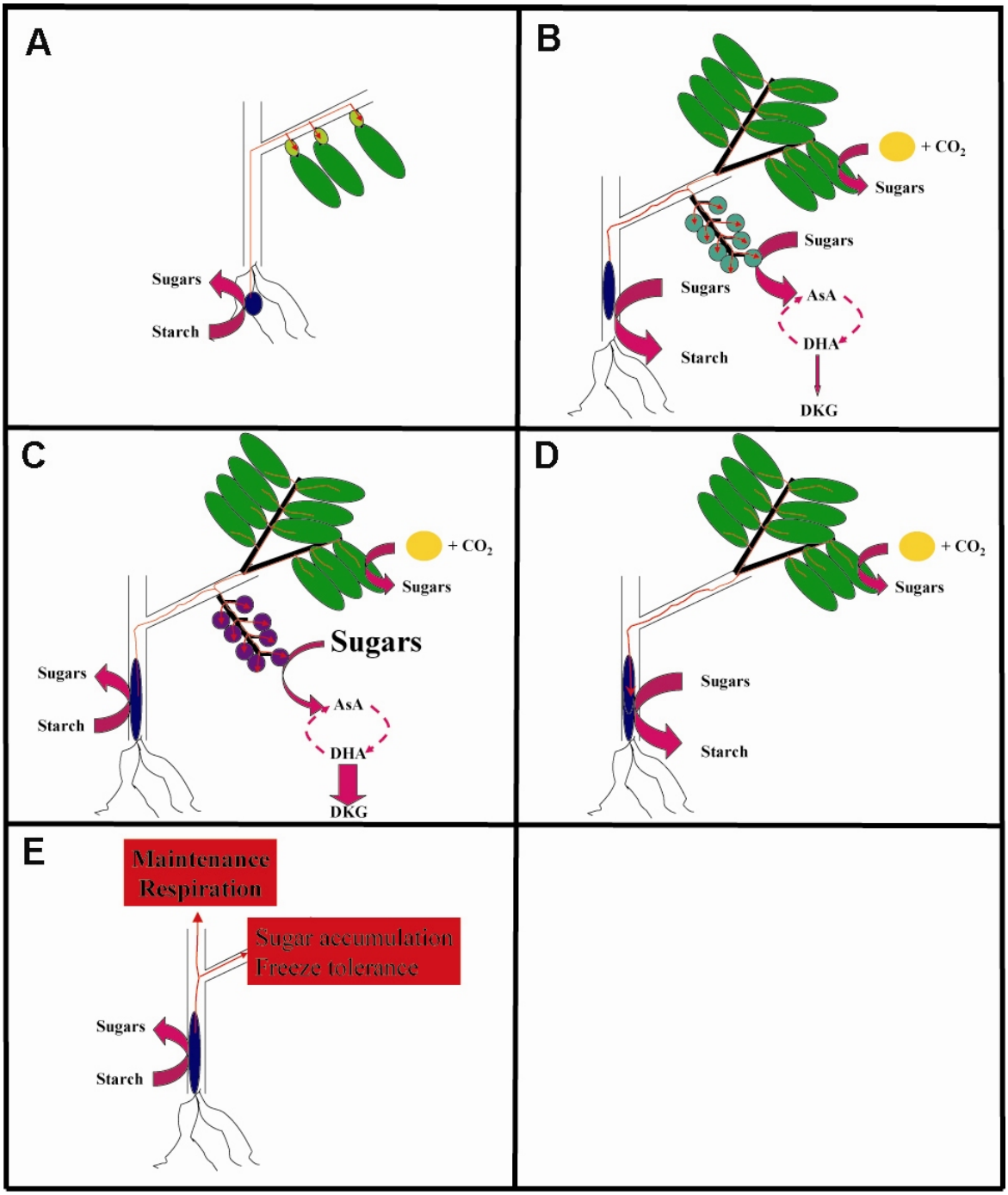


Figure 1.17 A model for AsA accumulation in blackcurrant fruit

Details are provided in the text. DHA = dehydroascorbic acid; DKG = diketogulonic acid

Objective 2 – Screen available germplasm and identify early correlative and predictive markers of AsA berry content

Rationale

In the longer term, fruit AsA concentration may be raised by selective breeding to generate novel high AsA cultivars. Additionally, to meet the ever changing needs to industry, novel blackcurrant cultivars must be continually developed that combine high fruit AsA concentrations with other desirable traits be they agronomic (e.g. yield, disease resistance), quality (e.g. flavour, colour) or processing (e.g. juice yield) traits. In order to meet these objectives a series of blackcurrant populations were screened for high AsA genotypes to identify novel germplasm to include in ongoing breeding programmes at SCRI and elsewhere. Furthermore, to accelerate current breeding programmes metabolic and genetic markers were sought to allow identification of high fruit AsA genotypes at the seedling stage. The use of such markers will allow the removal of unpromising lines at an early stage of development allowing increasing numbers of crosses to be made each year without the requirement for additional labour. The increased numbers of crosses each year will accelerate the development of blackcurrant genotypes combining an acceptable concentration of AsA with other desirable traits.

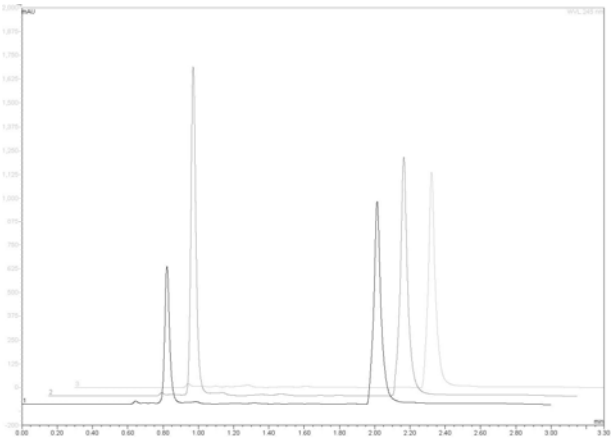
Headlines

- 300 individuals within the SCRI breeding programme sampled at random had fruit AsA concentrations between 0.56 and 3.64 mg gFW^{-1}
- A Hedda x 8872-1 cross had fruit with AsA concentrations between 1.06 and 4.63 mg g FW^{-1}
- Fruit AsA heritability was maternally influenced
- A mutagenised blackcurrant population had fruit with AsA concentrations between 0.82 and 2.71 mg g FW^{-1}
- A Ben Starav × Ben Gairn cross used for the determination of predictive metabolic markers had fruit with AsA concentrations between 0.60 and 3.25 mg g FW^{-1}
- Predictive metabolic markers could not be identified

- Gene sequence analysis of 4 biosynthetic genes in 35 blackcurrant genotypes revealed polymorphisms associated with high fruit AsA in the gene encoding GDP-D-mannose 3,5-epimerase

HPLC Method Development

In order to allow phenotyping of large blackcurrant populations, a novel method for HPLC estimation of fruit AsA concentrations was developed. The method allowed for the separation and quantification of AsA in fruit samples within 3 min (Fig. 2.1). The identity of AsA and purity of the peak was confirmed by (i) co-elution with authentic AsA, (ii) correlation of peak absorption spectrum with that of authentic AsA, (iii) enhancement of peak area following incubation of sample with the reducing agent TCEP and (iv) removal of peak following incubation of sample with ascorbate oxidase.



Time (min)

Figure 2.1 HPLC traces of Baldwin fruit extracts

Baldwin fruit were extracted in 5% metaphosphoric acid and injected onto a monolith C18 column (Phenomenex, UK) using 30 mM potassium phosphate pH 2.8 at 2.5 ml min^{-1} as mobile phase. Traces recorded UV absorbance at 245nm. Immediately after AsA had eluted (the peak at ~ 0.8 min), the column was washed with a gradient to 80% acetonitrile and then reequilibrated with potassium phosphate. Trace 1 – Crude extract, trace 2 – crude extract treated with 5 mM TCEP, trace 3 – crude extract treated with ascorbate oxidase.

AsA content of SCRI germplasm

The HPLC method developed was used to determine the AsA concentration in blackcurrant juice from 300 individuals in the SCRI breeding program. Fig. 2.2 shows the distribution of AsA concentration in the individuals tested. The majority of plants tested bore fruit with AsA concentrations less than 200 mg/100 ml juice (77%) however a small minority showed exceptional AsA concentrations greater than 300 mg/100 ml juice (1.3%) (Fig. 2.2).

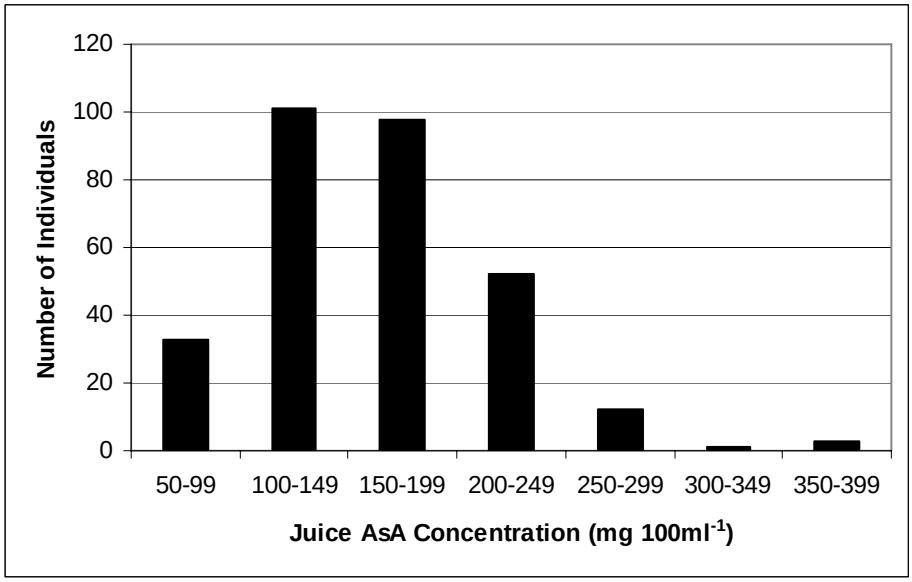


Figure 2.2 Distribution of AsA concentration in blackcurrant juice from 300 individuals in SCRI breeding program

The data outlined give a representation of the genetic diversity within the SCRI breeding programme and suggest that there is still scope for improving the fruit AsA concentration of commercial cultivars which is typically around 160 mg / 100 ml juice.

Inheritability of fruit AsA concentration

To obtain information regarding the heritability of fruit AsA concentration a population was generated in which cv. Hedda (fruit AsA concentration 67 mg / 100 ml juice) and genotype 8872-1 (272 mg / 100 ml juice) were crossed. Each genotype acted both as the donor and recipient of pollen in order to determine any paternal or maternal influence on fruit AsA heritability. Fig. 2.3 shows that progeny had a broad range of fruit AsA concentrations (106 – 463 mg / 100 ml juice) with both the lower and higher values

significantly exceeding the concentration of AsA found in Hedda fruit while the higher value also significantly exceeded that found in 8872-1 fruit. There did appear to be strong maternal influence on fruit AsA concentration with the top nine individuals in the 78 strong population being maternally related to 8872-1. These maternal effects may be related to the inheritance of organelle encoded DNA. In particular the final step in AsA biosynthesis is located at the inner mitochondrial membrane (Siendones *et al.*, 1999) and integrated into the electron transport chain (Bartoli *et al.*, 2000). Several components of the electron transport chain are encoded by mitochondrial DNA (Kubo and Mikami, 2007) and maternally inherited differences may affect the efficiency and control of AsA biosynthesis.

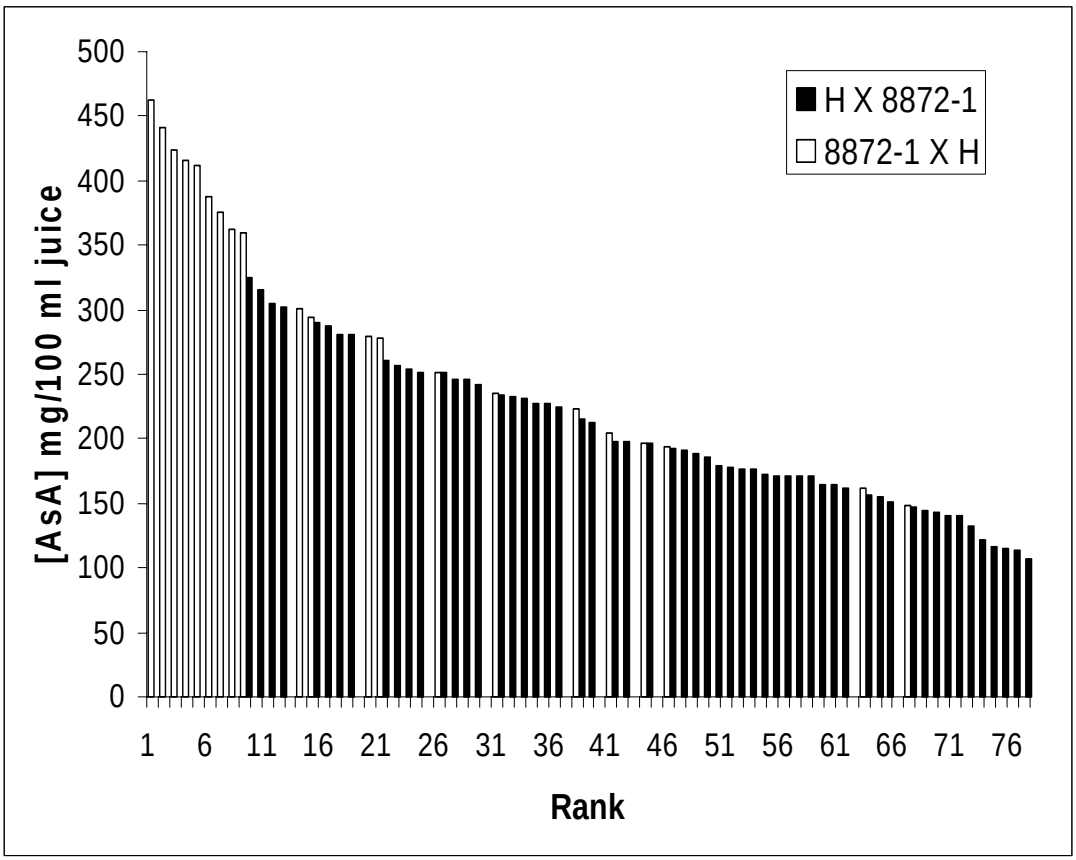


Figure 2.3 Heritability of fruit AsA concentration in blackcurrant

Crosses were made between cv. Hedda and genotype 8872-1 where 8872-1 acted as the pollen donor (H X 8872-1) or pollen recipient (8872-1 X H). Seedlings were grown on in the glasshouse prior to transfer to field conditions. Fruit were sampled four years after initial germination and AsA extracted and analysed as described in the text.

Impact of mutagenesis on fruit AsA concentration

In an attempt to extend the variation found within the germplasm available to the SCRI breeding programme, seeds produced from several crosses were subjected to gamma radiation at different doses in order to induce mutation. The data described in table 2.1 suggests that no useful mutations were induced as fruit subsequently harvested from mature plants grown from the irradiated seed failed to show any individuals with exceptionally high fruit AsA.

Population	**Ben Lomond selfed**			**Ben Avon × Ben More**		
Radiation Dose (Gy)	100	150	200	100	150	300
Number harvested	23	12	5	21	5	1
AsA Max (mg / 100 ml Juice)	271	223	191	271	161	165
AsA Min (mg / 100 ml juice)	82	96	119	105	118	165
AsA Mean (mg / 100 ml juice)	167	157	153	163	144	165
Standard Deviation	52	39	27	45	19	NA
Relative Standard Deviation (%)	31	25	18	27	13	NA

Table 2.1 Fruit AsA concentrations in blackcurrant populations grown from irradiated seed

Seeds from the crosses described were gamma irradiated with the dose described. Fruit were then germinated and grown on to maturity (number harvested). For each population, the maximum, minimum and mean fruit AsA concentrations at each radiation dose received. Population standard deviation about the mean and relative standard deviation is also provided. NA, not applicable.

Identification of predictive markers for fruit AsA concentration

Analysis of various blackcurrant populations at SCRI (see above) suggested that although there was reasonable variability within the SCRI breeding programme, there were not any exceptional genotypes containing very high levels of AsA in fruit (> 500 mg / 100 ml juice). Furthermore, during the course of the work programme, it became clear that the priorities for fruit quality were becoming broader with increasing demand for not only high AsA concentrations but also for enhanced total antioxidant capacity, improved flavour and optimal colour. In order to accelerate the development of cultivars with optimal agronomic and processing parameters, a change in project milestones were agreed and efforts were focussed on the development of metabolic or genetic predictive markers. The objective of the work was to allow the prediction of mature fruit AsA concentration from the measurement of a biological parameter in seedlings. Successful development of such markers would allow the removal of plants with unacceptably low predicted fruit AsA levels at the seedling stage providing efficiency savings both in terms of labour and land

use for the propagation of plants through to maturity. The expected result would be the possibility to generate increased crosses each year with no additional labour costs thus increasing the capacity for the development of cultivars combining acceptable fruit vitamin C levels with other desirable agronomic, processing and quality traits.

i. Biochemical Approach

A population of 400 seedlings from a Ben Starav × Ben Gairn cross was established at SCRI and leaf tissue sampled following acclimation of the plants to a controlled environment for one week. The plants were transferred to the field in January 2007 and harvested in August 2007. The AsA concentration of fruit was determined and correlated with several metabolites extracted from seedling tissues.

a. Ascorbic acid

Although AsA is likely to perform different functions in actively photosynthesising seedling leaves and ripening fruit, the level of AsA accumulation may be governed by similar mechanisms. For example, a genetic polymorphism in a biosynthetic enzyme may result in the expression of an enzyme with greater efficiency in both tissues which may lead to a plant with a higher than average AsA concentration in different tissues at different developmental stages. For this reason, AsA was measured in both seedling leaves and in mature fruit to determine the presence or absence of any correlation.

Fig. 2.4 shows a frequency chart of fruit AsA concentration in the 190 seedlings that fruited in the 2007 season. The data shows an approximate normal distribution with a mean fruit AsA concentration of 1.82 mg g FW^{-1} and minimum and maximum values of 0.60 and 3.25 mg g FW^{-1}, respectively. The standard deviation about the mean was 0.48 which represented a relative standard deviation of 26%. The population distribution and wide range between minimum and maximum values (5.5 fold) suggest a useful population for the determination of predictive markers.

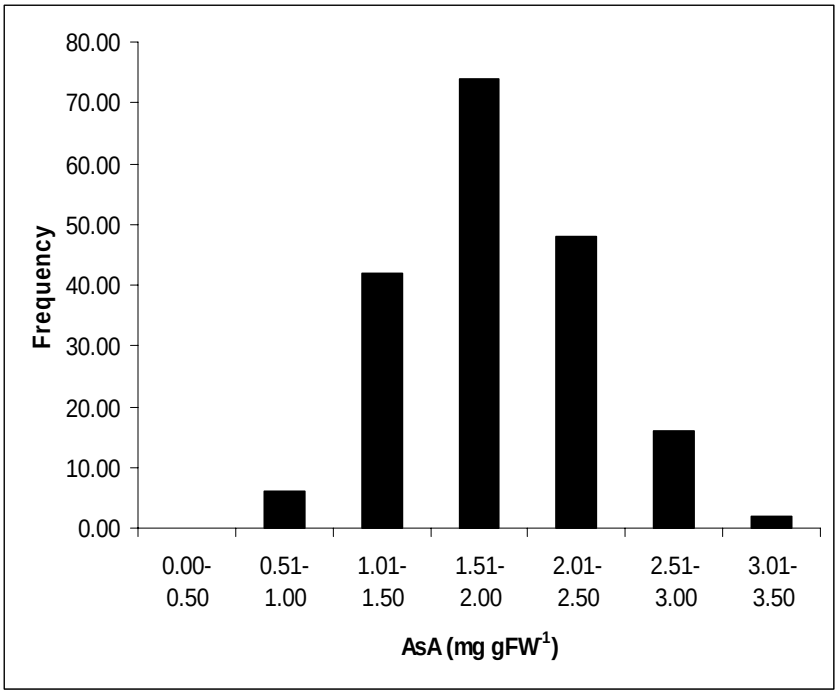


Figure 2.4 Frequency distribution of fruit AsA concentration in a Ben Starav × Ben Gairn seedling population

2 year plants were harvested in August 2007. Fruit AsA was extracted as described in the text and quantified by HPLC. AsA concentration of fruit on individual plants was determined as the mean of three separate fruit extractions.

The leaf AsA content of young seedlings also showed an approximately normal distribution with very close mean (3.12 mg g FW^{-1}) and median (3.09 mg g FW^{-1}) values. The standard deviation about the mean was 0.87 (% SD = 28%) and the minimal and maximal values were 0.49 and 6.14 mg g FW^{-1}, respectively representing a range of 12.5 times (Fig. 2.5). Correlations between seedling leaf and mature fruit AsA were low (Fig. 2.5) with an R^2 value below 0.05 and therefore seedling leaf AsA concentration can not be considered a suitable predictor of mature fruit AsA concentration in blackcurrant plants.

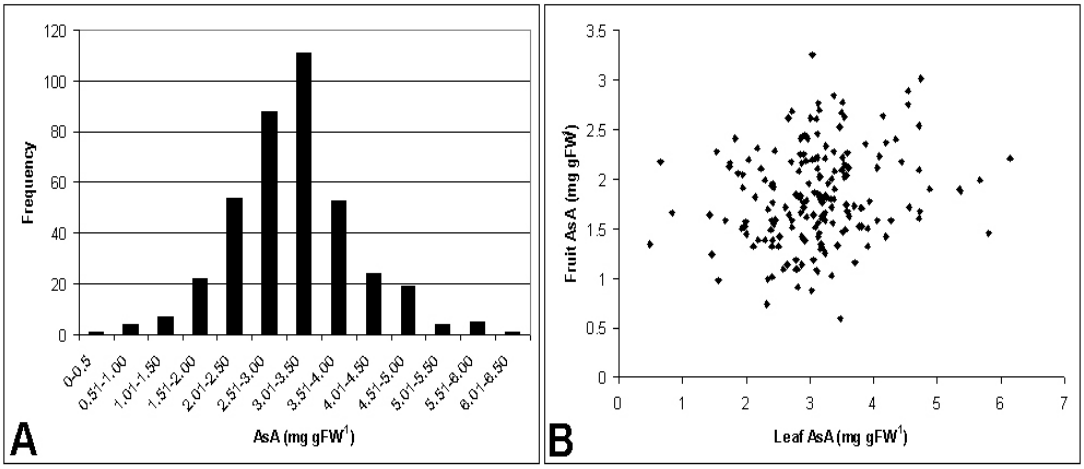


Figure 2.5 Correlation between seedling AsA concentration and mature fruit AsA concentration in a blackcurrant population

The third fully expanded leaf from 20 cm high seedlings was sampled following 1 week in a control environment chamber and AsA extracted and quantified as described in the text. Panel A, population distribution of leaf AsA concentration; Panel B – Correlation between seedling leaf and mature fruit AsA concentrations.

b. Organic acids

The tricarboxylic acid pathway (TCA) represents the primary of source NADH for the donation of electrons electrons into the respiratory electron transport chain. In addition, electrons can be directly donated from succinate, a key intermediate of the TCA. Therefore, a close relationship exists between intracellular organic acid concentration and respiratory activity. As the electron transport chain represents a significant source of hydrogen peroxide in plant cells (Foyer & Noctor, 2003) and the AsA consuming enzyme ascorbate peroxidase represents a significant mechanism of H_2O_2 detoxification there is a potential link between respiratory activity, organic acid concentration and AsA concentration. In addition, recent research has demonstrated that the ultimate enzyme in AsA biosynthesis, L-GalL dehydrogenase is intimately associated with components of the electron transfer chain (Bartoli *et al.*, 2000) and that respiration is involved in the control of AsA biosynthesis (Bartoli *et al.*, 2006). Experimental evidence in support of the relationship between organic acids, respiration and AsA concentration was presented by Nunes-Nesi and colleagues (2005) who generated tomato plants with reduced malate dehydrogenase activity which had elevated leaf succinate and AsA concentrations. Further

associations between organic acids and AsA exist as a consequence of AsA acting as a potential biosynthetic precursor to several organic acids including oxalate, threonate and tartrate (DeBolt *et al.*, 2007).

Seedling leaf organic acids were determined by HPLC in the same extract as AsA. Oxalic acid was identified by virtue of its coelution and identical absorption spectra compared with an authentic standard. Two other peaks (peaks 2 and 3) were identified that had absorption spectra consistent with those of organic acids (λ_{max} 210 and 214nm, respectively) although further identification was not possible. Frequency graphs (Fig. 2.6) revealed that the distribution of both oxalate and peak 2 concentrations within the leaves of the seedling populations approximated to a normal distribution as did that of peak 3 although this compound showed greater clustering around the mean.

Statistical parameters regarding the population distributions for AsA, oxalate, peak 2 and peak 3 concentrations are shown in table 2.2. The broad distribution of organic acid concentrations in seedling leaves suggest their potential utility as predictive markers. Within leaves, only very low correlation coefficients were observed between organic acid and AsA concentrations suggesting only weak association between organic acids, respiration and AsA synthesis.

Parameter	Oxalate	Peak 2	Peak 3	AsA
Mean	4.26	105.9	163.2	3.12
Median	4.16	99.4	163.8	3.09
SD	1.53	43.9	70.5	0.87
% SD	35.8	41.5	43.2	28.0
Minimum	0.73	8.1	15.2	0.49
Maximum	10.46	252.7	368.4	6.14
Q1	3.18	76.6	112.1	2.61
Q3	5.28	130.4	212.8	3.53
Range	14.3	31.1	24.2	12.5
R^2 vs. AsA	0.16	0.14	0.09	NA

Table 2.2 Statistical parameters regarding the distribution of organic acid concentration in leaves of a Ben Starav × Ben Gairn seedling population

Leaf discs were extracted in metaphosphoric acid and organic acid concentration determined by HPLC. Values are given as mg gFW^{-1} for oxalate and AsA and as arbitrary vales (peak area) for peaks 2 and 3. SD = standard deviation, Q1 = first quartile, Q3 = third quartile, R^2 vs. AsA = correlation coefficient for a plot of organic acid concentration against AsA concentration, a perfect correlation provides an R^2 of 1.00.

Correlations between seedling leaf organic acid concentration and mature fruit AsA concentrations were also low with R^2 values of 0.0003, 0.0171 and 0.004 for oxalic acid, unknown organic acid 1 and unknown organic acid 2, respectively. Hence seedling leaf organic acid concentrations were not suitable markers for the prediction of fruit AsA concentration in mature plants.

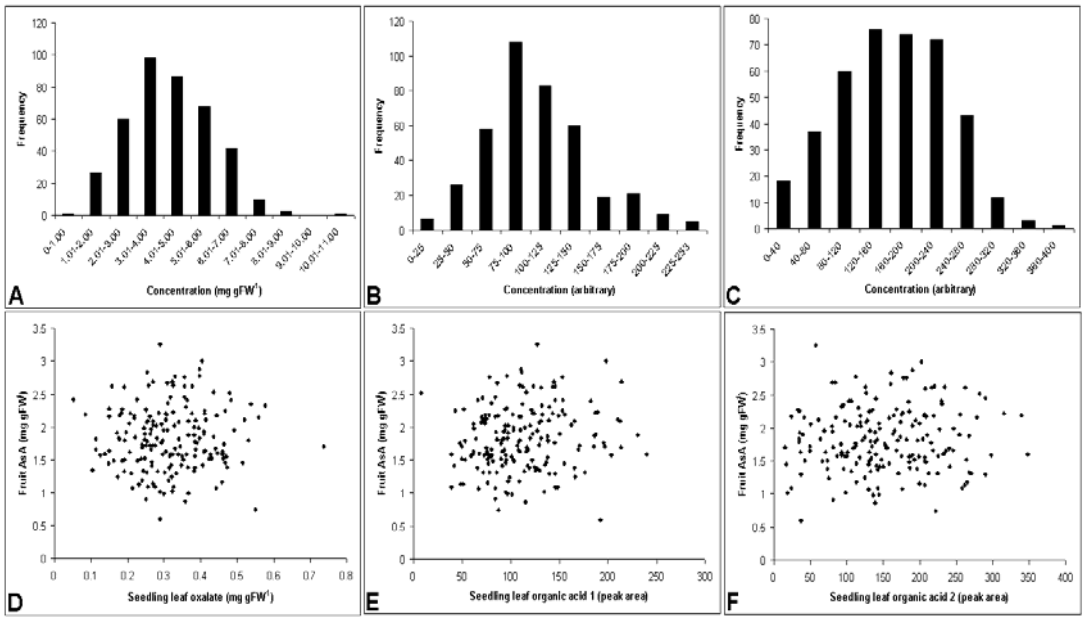


Figure 2.6 Frequency distribution of three putative organic acids in leaves of a Ben Starav × Ben Gairn seedling population and correlation with mature fruit AsA

Organic acids were extracted from leaf discs and fruit and analysed by HPLC as described in the text. Panels A, B and C, frequency distributions for concentration of oxalic acid, unknown organic acid 1 and unknown organic acid 2, respectively in leaf discs; panels D, E and F, correlations between leaf organic acid and fruit AsA for oxalic acid, unknown organic acid 1 and unknown organic acid 2, respectively.

c. Glutathione

Glutathione (GSH) and AsA represent the two major soluble antioxidant pools in plant cells (Foyer and Noctor, 2005). Furthermore, GSH plays a crucial role in recycling oxidised AsA via the ascorbate-glutathione cycle (Hancock and Viola, 2005). The importance of AsA recycling has been demonstrated in transgenic plants where the overexpression of dehydroascorbate reductase, an enzyme capable of reducing dehydroascorbate to AsA using GSH as a cofactor, resulted in up to 4-fold higher AsA (Chen *et al.*, 2003). This modification also resulted in an increase on the plant GSH content up to 2.6-fold. These data suggest a correlation between the levels of glutathione

and AsA and further evidence for this link was provided by a similar approach in which GSH levels were doubled in poplar by overexpression of a gene encoding glutathione reductase, responsible for recycling oxidised GSH (Foyer *et al.*, 1995). In these plants, AsA concentrations were also doubled.

GSH was determined in the same seedling leaf extracts as AsA and other organic acids using HPLC coupled with electrochemical detection. Unlike the organic acids, GSH was towards lower values within the seedling population although a small number of individuals did have relatively high leaf GSH concentrations (Fig. 2.7). The range of leaf GSH concentrations within seedlings was very broad with a minimum concentration of 0.04 mg g FW^{-1} and a maximum of 1.35 mg g FW^{-1}. The broad range of values was reflected in the large standard deviation of 0.20 about a mean leaf GSH concentration of 0.41 mg g FW^{-1}. Despite the proven links between GSH and AsA within a single tissue (see above), the correlation between seedling leaf GSH concentration and mature fruit AsA concentration was very poor (Fig. 2.7) with an R^2 value of only 0.0038.

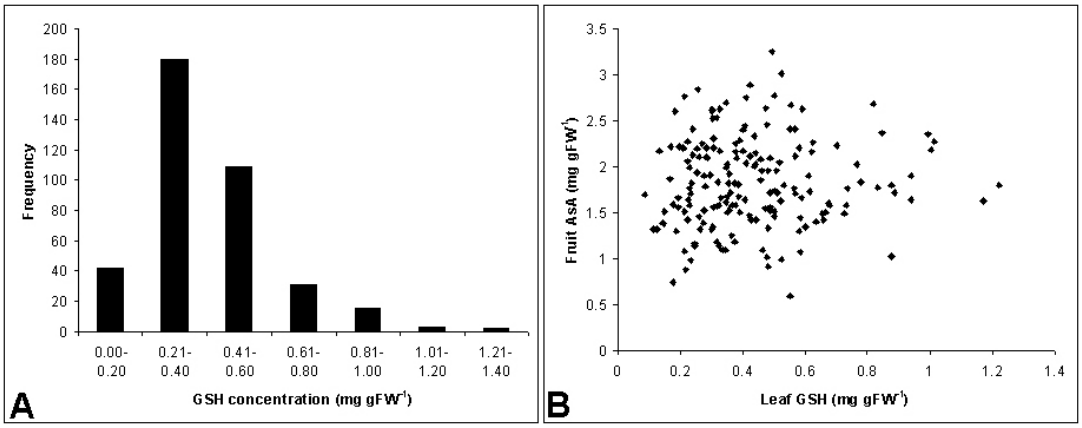


Figure 2.7 Frequency distribution of blackcurrant seedling leaf GSH concentration and correlation with mature AsA concentration

Leaf GSH and fruit AsA were quantified by HPLC with coulimetric and diode array detection, respectively as described in the text. Panel A, frequency distribution of leaf GSH concentration; panel B, correlation between leaf GSH concentration and fruit AsA concentration.

d. Antioxidant capacity

There is a growing recognition of the interdependence of antioxidant pools within plant tissues (Foyer & Noctor, 2005; Noctor, 2006) and it has been shown at least *in vitro* that several phenolic antioxidant compounds have the capacity to maintain AsA in its reduced

state (Millar & Rice-Evans, 1997). Therefore plants which maintain a generally high level of antioxidant compounds might also maintain a high level of AsA.

Leaf discs were sequentially extracted in water, 50% methanol and acidified 50% methanol to release the aqueous, lipophillic (membrane soluble) and wall bound antioxidant fractions, respectively. The total antioxidant capacity of each fraction was estimated using Folin-Ciocalteu reagent as described in materials and methods and summed to give the total antioxidant capacity within the leaf. The majority of leaf antioxidant capacity was associated with the aqueous fraction which represented about 60% of the total while lipophillic and bound fractions represented 25% and 15% respectively (table 2.3).

Parameter	Total	Aqueous	Lipophillic	Bound
Mean	31.42	18.56	7.62	5.24
Median	26.76	14.89	6.37	4.37
SD	19.45	14.69	5.14	3.27
%SD	61.92	79.17	67.41	62.37
Minimum	3.77	1.23	0.67	0.36
Maximum	128.56	102.67	28.12	19.48
Q1	17.01	8.48	3.79	2.79
Q3	39.96	23.98	10.12	7.01
Range	34.06	83.27	41.74	62.37
R^2 vs. AsA	0.06	0.06	0.06	0.00

Table 2.3 Statistical parameters regarding the distribution of antioxidant capacity in leaves of a Ben Starav × Ben Gairn seedling population

Leaf discs were sequentially extracted in water, 50% methanol and acidified 50% methanol and total antioxidant capacity determined spectrophotometrically. Values are given as mg gFW^{-1} trolox equivalents. SD = standard deviation, Q1 = first quartile, Q3 = third quartile, R^2 vs. AsA = correlation coefficient for a plot of organic acid concentration against AsA concentration.

For all fractions tested, the range of concentration was much greater than that observed for organic acids (table 2.2) and the distribution frequency was skewed towards the lower end with a few outliers containing very high levels of antioxidants (Fig. 2.8) as was further illustrated by the low median and first quartile values.

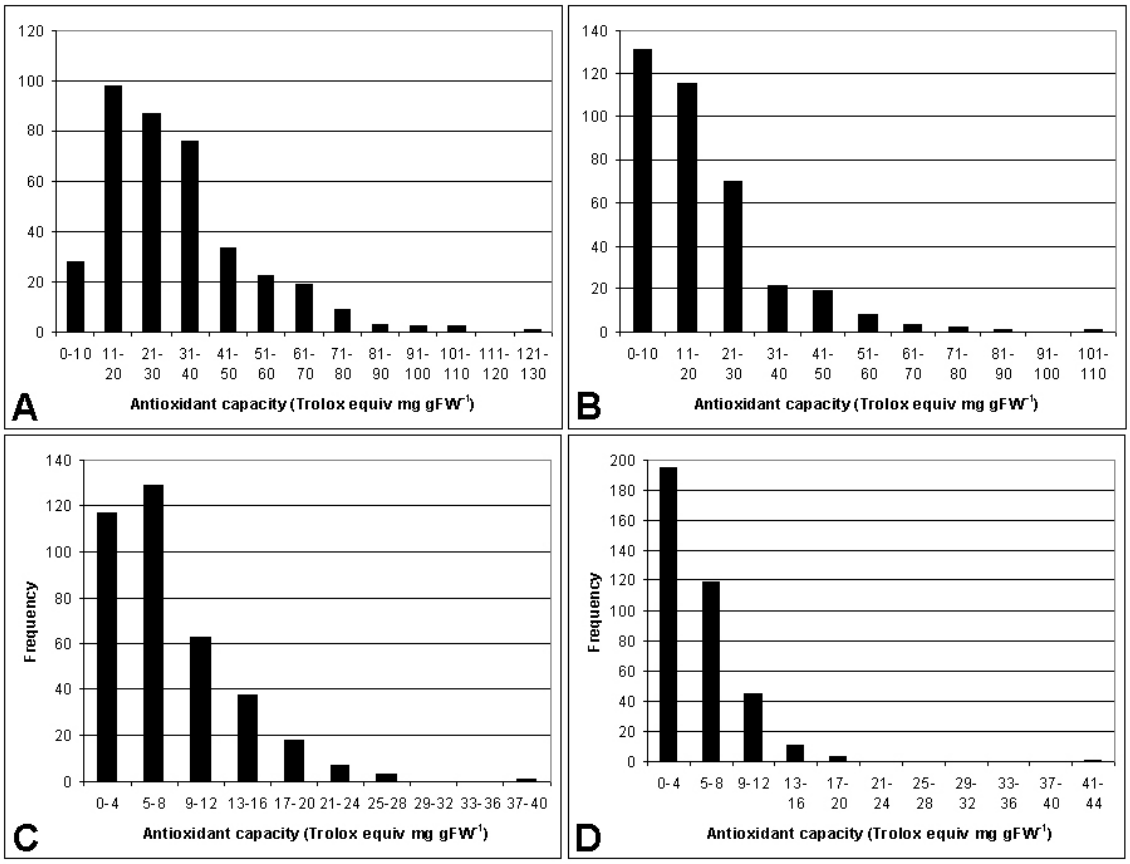


Figure 2.8 Frequency distribution of antioxidant fractions in leaves of a Ben Starav x Ben Gairn seedling population

Aqueous, lipophillic and bound antioxidants were extracted from leaf discs and analysed spectrophotometrically as described in materials and methods. Total antioxidant capacity was determined by summation. Panel A, total antioxidant capacity; B, aqueous antioxidant capacity; C, lipophillic antioxidant capacity; D, bound antioxidant capacity.

Correlations between all of the antioxidant fractions in seedling leaves and fruit AsA concentrations were exceedingly low (Fig. 2.9) with R^2 values below 0.01. Seedling leaf antioxidant capacity was therefore considered an unsuitable predictive marker for fruit AsA concentration in mature plants.

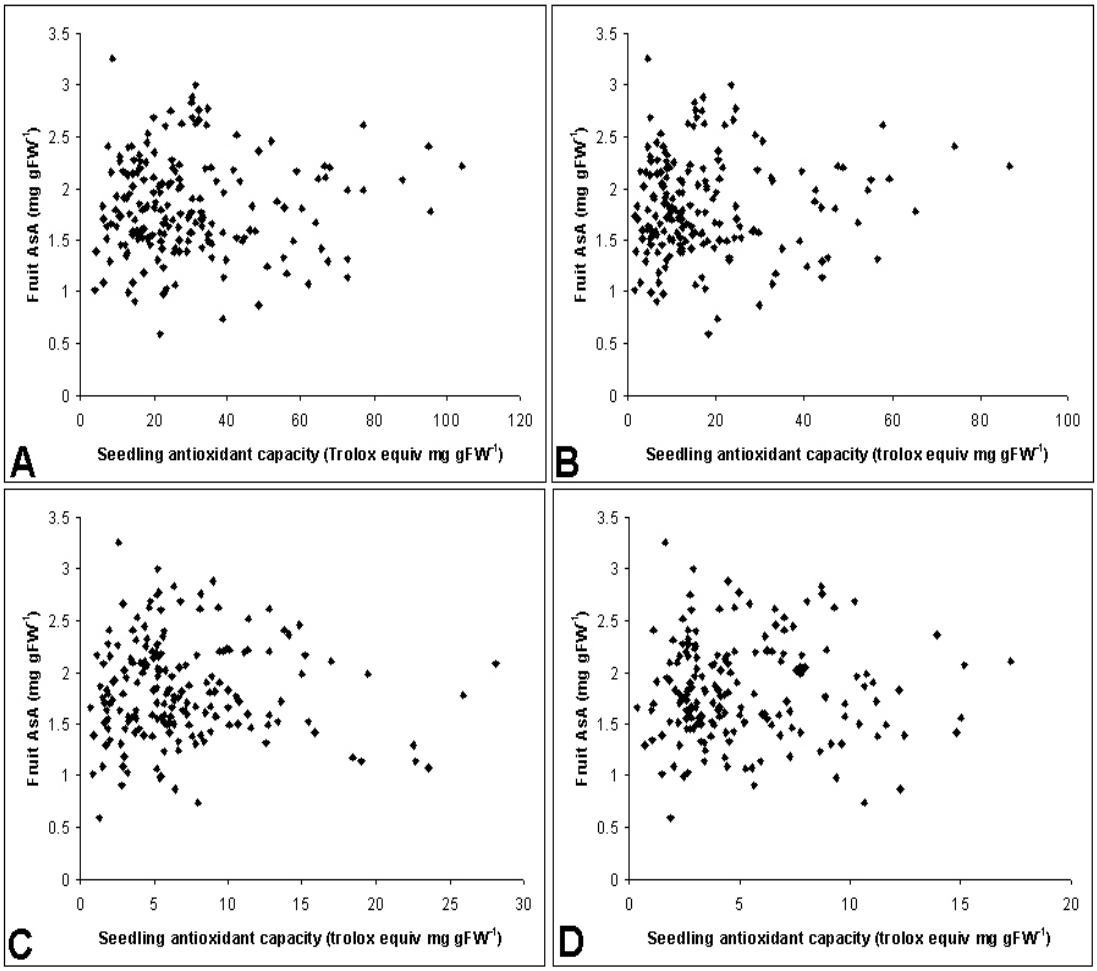


Figure 2.9 Correlation between seedling leaf antioxidant capacity and fruit AsA concentration in blackcurrant plants

Seedling leaf antioxidant capacity and fruit AsA concentrations were measured as described in the text correlations shown are between fruit AsA concentration and leaf total (panel A), hydrophillic (panel B), lipophillic (panel C) and wall-bound (panel D) antioxidant fractions.

e. Metabolite profile

In order to achieve the objective of developing markers for the prediction of fruit AsA content it would not be necessary to know the identity of the compound on condition that its concentration could be accurately recorded. Therefore HPLC/MS methods were developed in to allow the separation and quantification of as many metabolites extracted from seedling leaf discs as possible. Fig. 2.10 shows a typical chromatogram where in excess of 25 peaks were resolved by mass spectrophotometric detection. Several of these peaks contained multiple compounds as determined by analysis of the peak mass spectrum therefore a total of 39 compounds were quantified from each seedling extract.

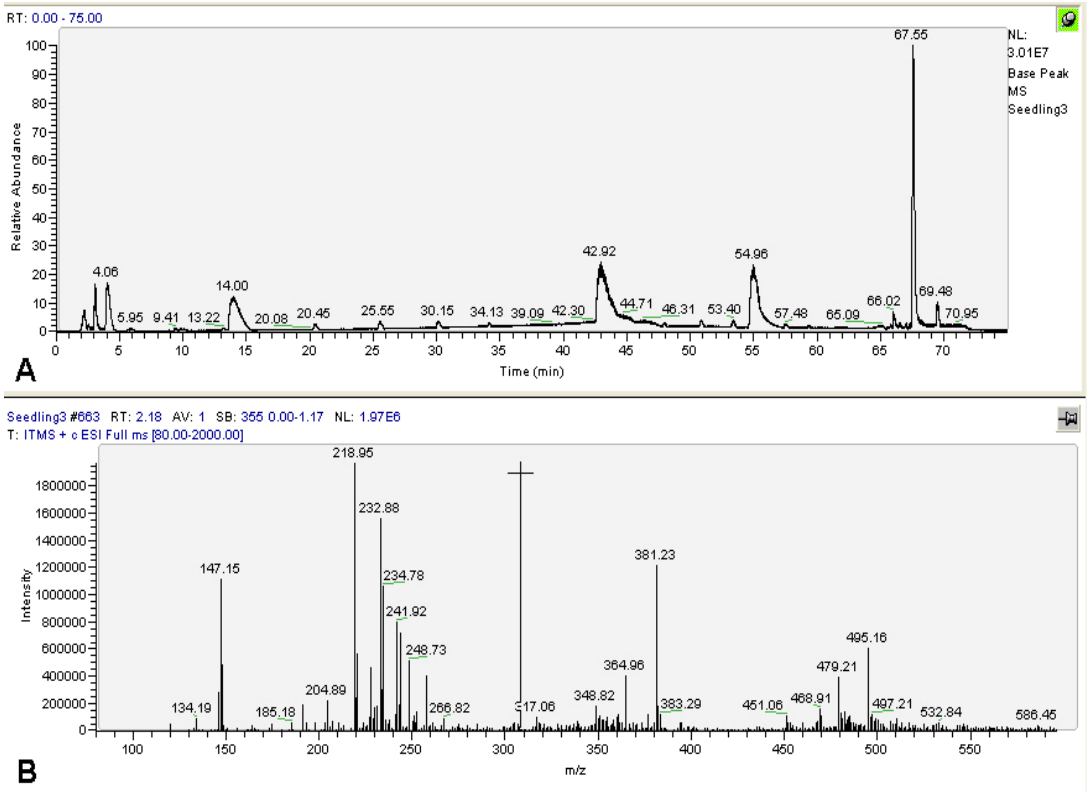


Figure 2.10 Metabolite profile of leaf extracts from blackcurrant seedling

Blackcurrant seedling leaf discs were extracted as described in materials and methods and metabolites were separated by HPLC using a methanol gradient. In excess of 25 individual peaks were observed by analysis of the base peak using mass spectrometry (panel A) and several of the peaks contained multiple compounds as determined by examination of their mass spectra. Panel B shows the mass spectrum of the peak eluting at 2.18 min and shows an abundance of compounds with different mass to charge ratios.

Table 2.4 shows the results of correlation analysis between metabolites extracted from seedling leaves and quantified by HPLC/MS and the AsA concentration in fruit of the same plants after reaching maturity. A wide range of values were observed for almost all of the metabolites identified with large standard deviation values often several times in excess of the mean. In general correlations between leaf metabolites and fruit AsA were low and a suitable predictive marker for fruit AsA content was not identified.

Metabolite Identification		Response Ratio		Peak Area	
Retention time (min)	**m/z**	**Mean ± SD**	**Correlation coefficient**	**Mean ± SD**	**Correlation coefficient**
2.18	219, 233, 235, 381, 495	248 ± 705	0.0557	1442 ± 2147	0.1220
2.56	177, 192, 409	21 ± 46	0.0682	130 ± 225	0.1855
3.02	298, 572	18 ± 48	0.0454	72 ± 76	0.2107
3.98	200, 398	125 ± 273	0.0670	1069 ± 2152	0.0806
12.54	163, 321, 343, 419, 619	17 ± 46	0.0542	56 ± 76	0.1605
13.06	223	268 ± 606	0.0468	2113 ± 4108	0.0833
13.17	133, 223	275 ± 613	0.0472	2115 ± 3982	0.0860
14.14	633	9 ± 24	0.0463	46 ± 27	0.0123
18.94	377, 399	15 ± 40	0.0531	73 ± 81	0.0830
26.44	361, 383, 459	10 ± 30	0.0541	48 ± 65	0.1304
27.51	419	1 ± 8	0.0176	5 ± 4	0.1374
40.05	569	43 ± 80	0.0414	287 ± 345	0.1127
42.9	613	29 ± 79	0.0339	174 ± 237	0.1079
45.37	658	17 ± 59	0.0326	112 ± 197	0.0985
47.62	471, 702	14 ± 57	0.0332	80 ± 183	0.1180
49.84	229, 746	4 ± 16	0.0324	30 ± 116	0.0997
54.45	549	25 ± 52	0.0021	344 ± 620	0.0123
56.43	593, 695	24 ± 66	0.0011	300 ± 494	0.0251
58.21	637	18 ± 65	0.0010	200 ± 325	0.0336
59.85	682	11 ± 49	0.0007	111 ± 181	0.0399
67.31	301, 414	881 ± 3832	0.0123	2075 ± 1116	0.0236

Table 2.4 Correlation coefficients between seedling leaf metabolites and mature fruit AsA in an experimental blackcurrant population

Metabolites were identified according to their retention time and mass to charge ratio (m/z). Metabolite concentrations were calculated relative to the concentration of reserpine added as an internal standard (response ratio) and in terms of absolute concentration (peak area). For each metabolite both the mean value and standard deviation (SD) are presented as well as the correlation between metabolite concentration in seedling leaves and mature fruit AsA concentration.

f. Enzyme activities

Initial attempts to quantify L-galactose dehydrogenase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase from frozen blackcurrant leaves by spectrophotometry as described in materials failed as no activity was recovered from leaf extracts. On the contrary, active enzymes were successfully extracted from potato leaves using the same extraction methodology suggesting that either very low activities were

present in blackcurrant seedling leaves or that blackcurrant leaves contained specific compounds preventing the recovery of enzyme activity *in vitro*.

To improve assay sensitivity, a new approach was adopted in which enzyme activities from leaf extracts were determined using a recycling assay to quantify a reaction product (Gibon *et al*., 2004). The use of the recycling assay amplifies the signal improving sensitivity. In all cases, the assay protocol followed the same methodology. In the first step, appropriate substrates were incubated with crude leaf extracts in order to allow measurement of a specific enzyme activity associated with AsA metabolism. Following a set period of time, the reaction was stopped and if necessary, coupling reactions were allowed to proceed to convert products of the initial reaction to compounds that could be measured using a cyclic assay. In the second step, products of the initial reaction were isolated and in the final step, a cyclic reaction was started to measure those products. The rate of the cyclic reaction was dependent on the quantity of product produced in the initial reaction. Fig. 2.11 shows schematic representations of the reaction series for the measurement of GDP-Man pyrophosphorylase, L-Gal dehydrogenase, monodehydroascorbate reductase and glutathione reductase.

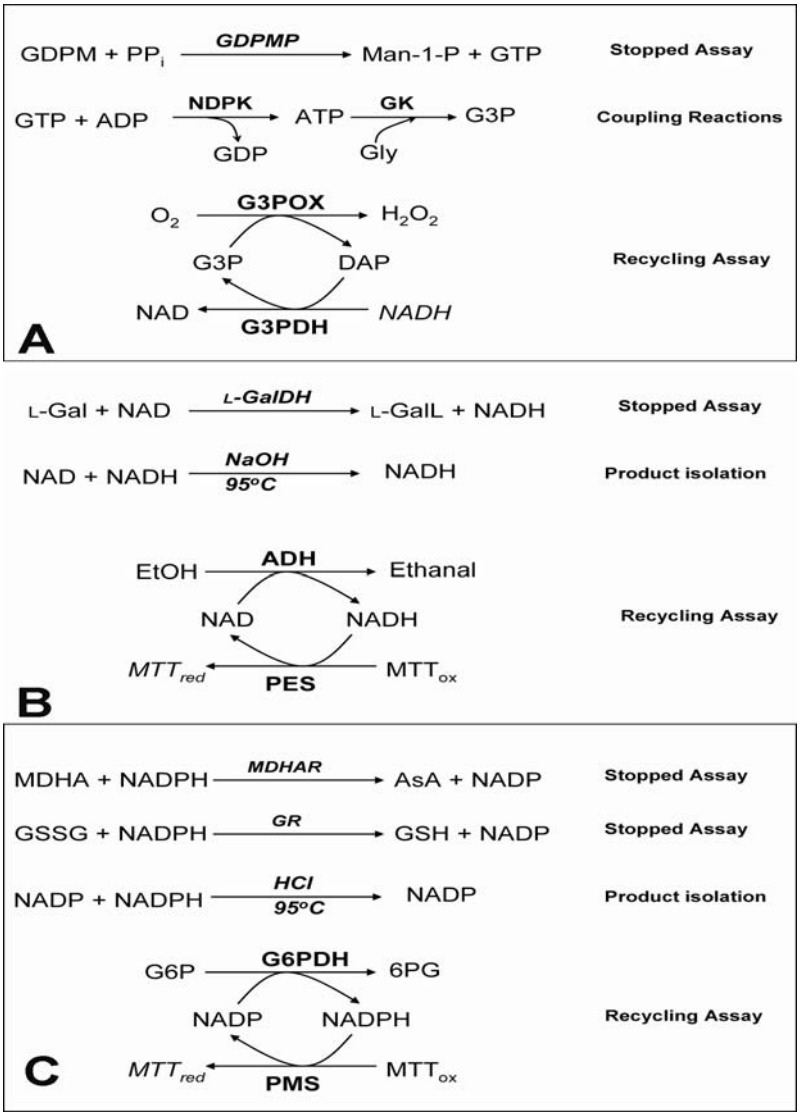


Figure 2.11 Schematic of reaction schemes for quantification of blackcurrant enzymes involved in AsA metabolism

Schemes for the quantification of GDP-mannose pyrophosohorylase (GDPMP; panel A), L-galactose dehydrogenase (L-GalDH; panel B), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR; panel C) are shown. For GDPMP quantification, GDP produced in a stopped reaction was quantitatively converted to G3P by means of a series of coupling reactions and finally G3P was indirectly measured in a recycling assay as the rate of conversion of NADH to NAD recorded by a loss of absorbance at 340nm. L-GalDH was quantified by measuring the amount of NADH produced following incubation of leaf extracts with L-Gal and NAD. NADH was measured as the rate of production of reduced MTT (λ = 570 nm) following removal of NAD by heating at 95^{o}C in the presence of 100 mM NaOH. MDHAR and GR activities were determined by measuring the production of NADP in the appropriate stopped assay. Following completion of the stopped assay, excess NADPH was removed by heating at 95^{o}C in 100 mM HCl and the NADPH concentration was estimated as the rate of production of reduced MTT in the appropriate recycling assay. Abbreviations: 6PG, 6-phosphogluconate; DAP, dihydroxyacetone phosphate; EtOH, ethanol; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; G3PDH, glycerol-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; G3POX, glycerol-3-phosphate

peroxidase; L-Gal, L-galactose; L-GalL, L-galactono-1,4-lactone; GDPM, GDP-mannose; GK, glycerol kinase; Gly, glycerol; GSH, reduced glutathione; GSSG, oxidised glutathione; Man-1-P, mannose-1-phosphate; MDHA, monodehydroascorbic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NDPK, nucleoside diphosphate kinase, PES, phenazine ethosuphate; PMS, phenazine methosulphate; PP_i, inorganic pyrophosphate.

In order to develop the cyclic assay technology, the initial stage was to determine the linearity and sensitivity of the cyclic assays for the measurement of reaction products. Fig. 2.12 shows linearity data for cyclic assays for the determination of G3P, NADH and NADP. For G3P, the limit of detection was 10 μM and the assay was linear up to at least 200 μM. Sensitivity and linearity for NADH was in a similar range (1 μM limit of detection, linearity to 20 μM) while sensitivity for NADP was significantly greater (down to 10 nM) and linearity was maintained to at 2 μM.

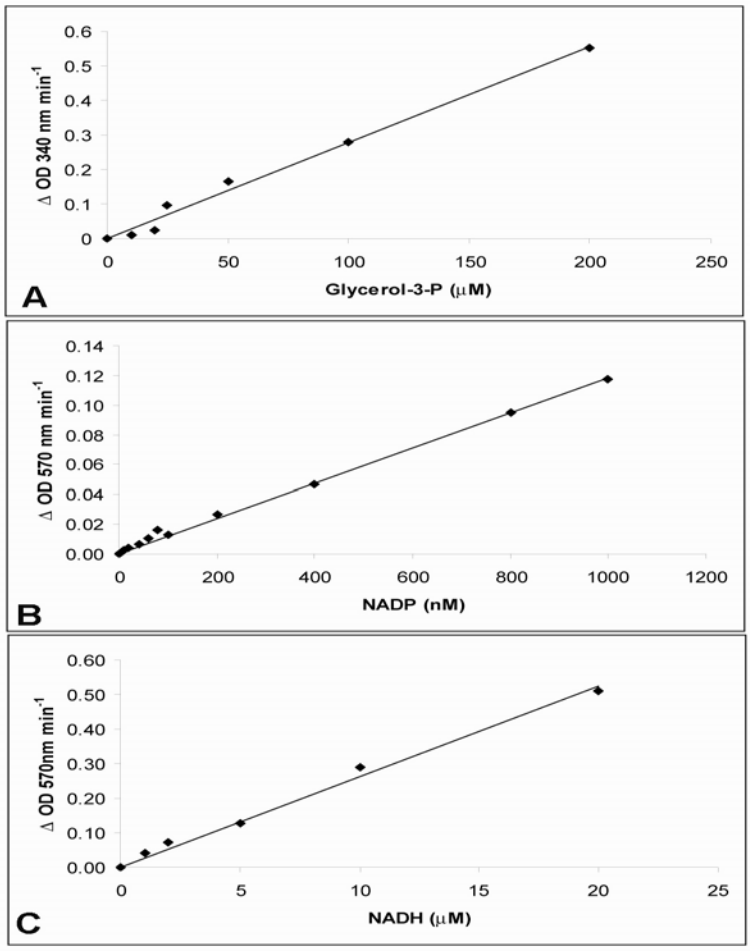


Figure 2.12 Linearity of response to reaction products in cyclic assays

Enzyme assays were undertaken as describe by Gibon et al. (2004). Substrates were G3P (panel A), NADP (panel B) and NADH (panel C).

A series of experiments to determine the efficiency of substrate destruction (NAD/NADPH) following heating in alkali or acid as appropriate were undertaken. The data presented in Fig. 2.13 shows that while NADH is unstable under acid conditions, heating for up to 6 min in 100 mM acid was insufficient to destroy all of the NADH present in the sample and approximately 20% of the original sample remained. Under the same conditions, NADP showed greater stability however, heating for as little as 1.5 min resulted in losses of approximately 20% and losses increased as heating time was extended. On the contrary, NADP was extremely unstable following heating under alkaline conditions and almost all was destroyed after 1.5 min. Under the same conditions, NADH showed excellent stability for up to 6 min (Fig. 2.13). These data suggest that the measurement of NADP production in stopped assays would be unsuitable as the excess NADPH present in the mixture would not all be destroyed and hence the cycling assays would give unreliable results. For this reason MDHAR and GR activities was not measured in blackcurrant leaves.

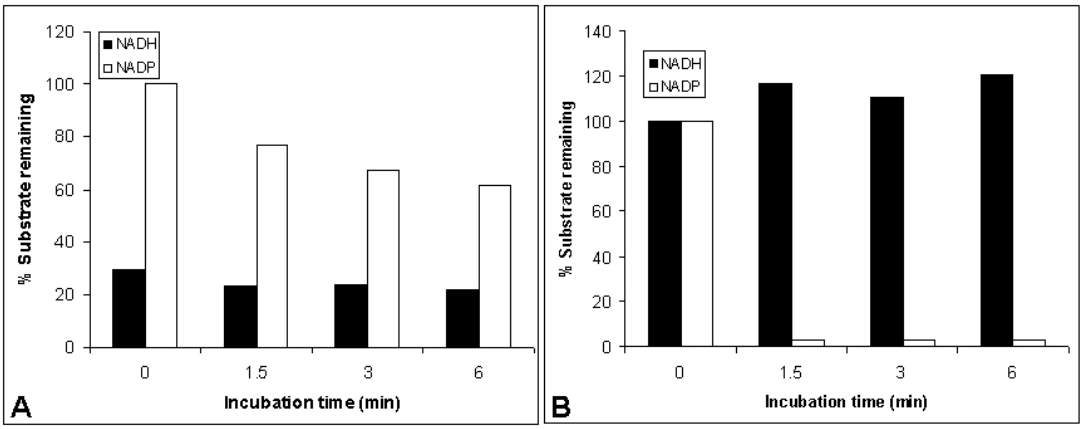


Figure 2.13 Stability of NADH and NADP at elevated temperature in acid or alkali

NADP (1 μM) or NADH (10 μM) were dissolved into 100 mM HCl (panel A) or NaOH (panel B), transferred to sealed vessels and heated for the appropriate time at 95°C. Samples were immediately cooled on ice and the remaining compound determined using the cycling assays described by Gibon et al. (2004).

Despite repeated attempts, we were unable to measure GDPMP activity in blackcurrant leaves, possibly as a result of the complexity of the coupling reactions and the different pH optima of nucleoside diphosphate kinase (pH 7-7.5) and glycerol kinase (pH 9-9.8). Although L-Gal dehydrogenase activity was detected, there was a lack of linearity between the quantity of enzyme extract used and the synthesis of NADH (data not shown) we were

therefore also unable to accurately quantify this enzyme activity in blackcurrant leaf extracts.

ii. Molecular Approach

Work described under objective 1 clearly showed that AsA accumulation in fruit was correlated with fruit biosynthetic capacity via the L-galactose pathway. In order to determine the rate limiting steps attempts were made to quantify biosynthetic enzyme activities in fruit of different cultivars at different stages of development. Despite a number of modifications to extraction procedures, consistent quantification of enzyme activities was not possible. To overcome these problems it was proposed that a molecular analysis could be used. Two objectives were outlined; to identify differences in gene expression between cultivars and stages of fruit maturity that might contribute to differences in biosynthetic capacity and to identify differences in coding sequence that might result in altered enzyme efficiencies and hence differences in biosynthetic capacity. Differences in gene expression may be linked to genetic polymorphism in the promoter regions of the gene which could be used as predictive molecular markers for fruit AsA concentrations. Alternatively, genetic polymorphism in the coding region may be related to enzyme efficiency and hence also provide predictive molecular markers for fruit AsA concentrations.

a. Gene expression analysis

Levels of gene expression in different cultivars at different stages of maturity were estimated by quantitative reverse transcription polymerase chain reaction (RT-PCR). As biochemical analyses suggested that fruit AsA accumulation was correlated with biosynthetic capacity, work was focussed on measurement of the expression of genes encoding four critical steps in the AsA biosynthetic pathway; GDP-mannose pyrophosphorylase (GDPMP), GDP-mannose 3,5-epimerase (GDPME), GDP-L-galactose phosphorylase (GDPGP) and L-galactose-1-phosphate phosphatase (GalP). None of the genes examined showed an expression pattern that correlated with biosynthetic capacity either between cultivar or between stages of fruit development (Fig. 2.14) suggesting that differences in gene expression were not responsible for differences in ripe fruit AsA concentration.

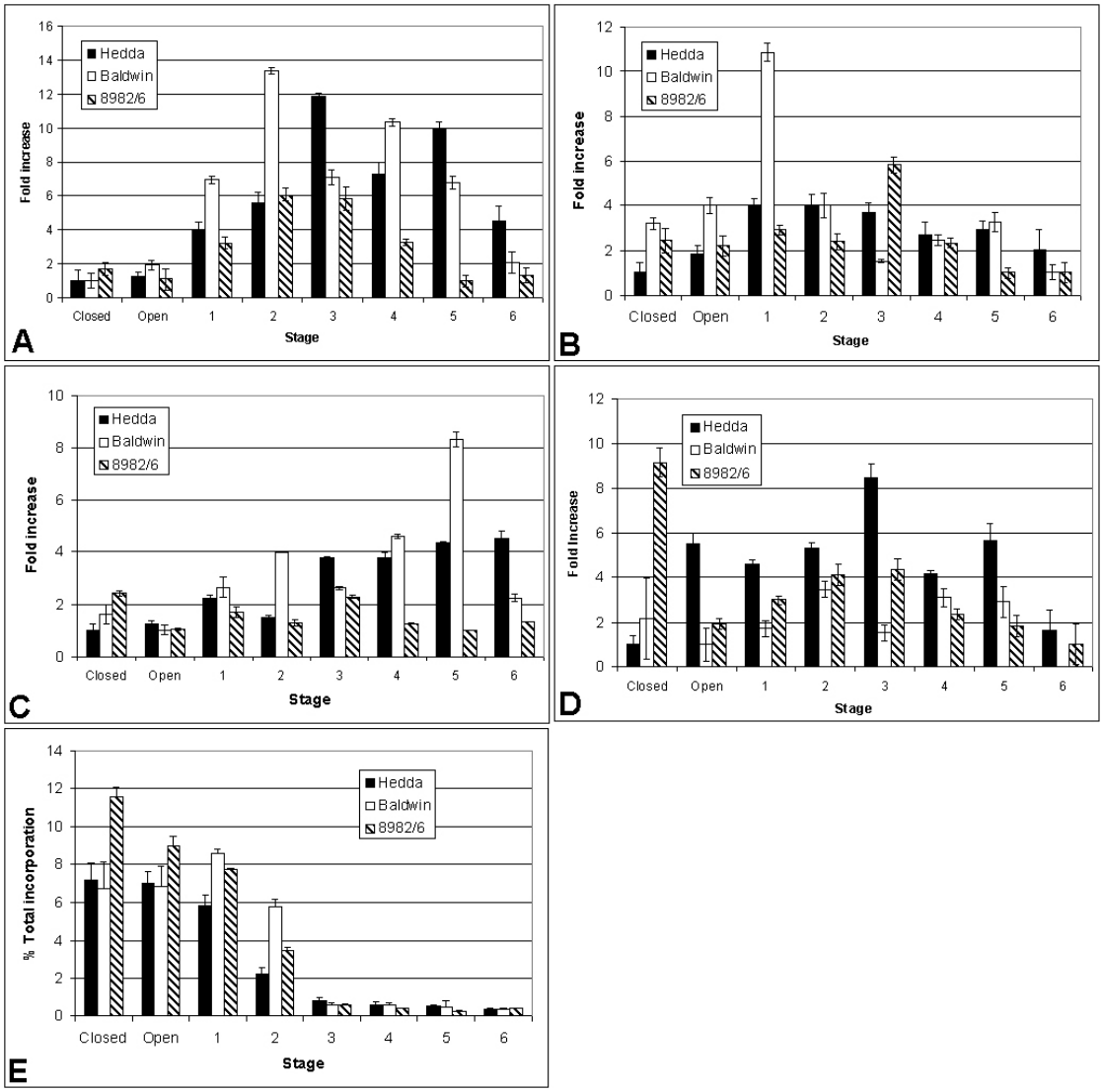


Figure 2.14 Expression of genes encoding AsA biosynthetic enzymes in fruit of three blackcurrant genotypes at different stages of development

Gene expression was estimated using quantitative RT-PCR as described in materials and methods using 18S rRNA as a reference. Fruit developmental stages were closed, closed flowers; open, open flowers; 1, small green fruit < 6 mm diameter; 2, large green fruit > 6mm diameter; 3, green-red fruit; 4, red-green fruit; 5, red fruit; 6, mature black fruit. Panel A, GDPMP; B, GDPME; C, GDPGP; D, GalP; E, biosynthetic capacity as determined by the partitioning of [U-^{14}C] mannose to [^{14}C]AsA.

In the three genotypes examined, GDPMP expression was low in flowers then increased in developing fruit before declining again as the fruit ripened. GDPMP not only participates in AsA biosynthesis but is also required to supply mannose residues for cell wall polysaccharides. This aspect of its function may account for the observed increase in gene expression during fruit expansion when cell wall mannose requirements are likely to be

high. GDPME participates in the generation of L-galactose for both AsA biosynthesis and for inclusion in cell walls. With the exception of stage 1 Baldwin fruit, changes in expression throughout fruit development were significantly less than those observed for GDPMP and failed to correlate with AsA biosynthetic capacity. GDPGP and GalP are not believed to have functions other than AsA biosynthesis however their patterns of expression also failed to correlate with AsA biosynthetic capacity in fruit.

b. Genetic polymorphism

Overlapping primers were designed in order to clone and sequence the full coding sequences for genes encoding GDP-mannose pyrophosphorylase, GDP-mannose 3,5-epimerase, GDP-L-galactose phosphorylase and L-galactose-1-phosphate phosphatase. Although several different primers were designed for the cloning of genes encoding the latter two enzymes sequence analysis of cloned fragments suggested that it was not the target genes that had been cloned. Fragments of the genes encoding both GDP-mannose pyrophosphorylase and GDP-mannose 3,5-epimerase were cloned from up to 36 different genotypes with fruit AsA concentrations ranging from 49 – 330 mg/100 ml juice.

Very little sequence variation was observed between genotypes in the gene encoding GDP-mannose pyrophosphorylase (Fig. 2.15) and there was no association between high AsA phenotypes and specific sequence variation suggesting that differences in coding sequence were unrelated to fruit AsA concentration.

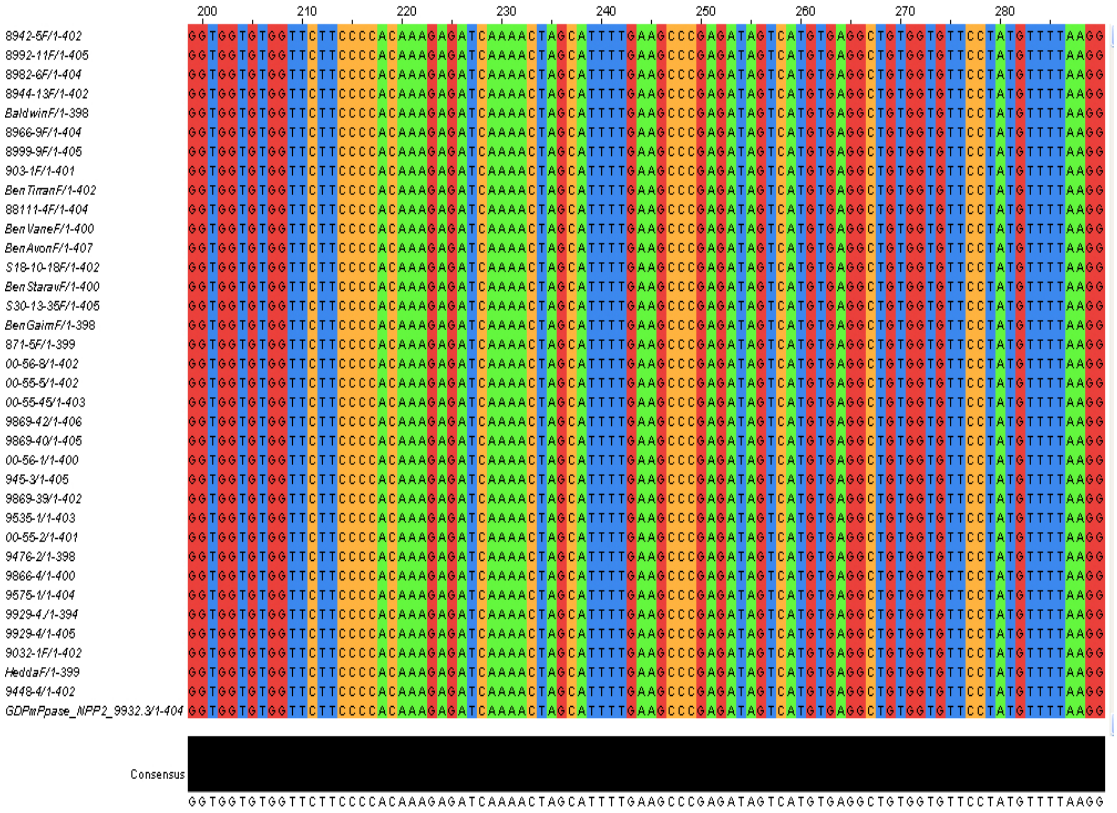


Figure 2.15 Alignment of a portion of the gene encoding GDP-mannose pyrophosphorylase from several blackcurrant genotypes

The GDP-mannose pyrophosphorylase encoding gene was cloned from cDNA of several blackcurrant genotypes containing a known fruit AsA concentration. Gene sequencing was performed and sequences aligned using the clustal program within Jalview. Genotype names are provided to the left of sequence and sequence are presented in descending order of fruit AsA concentration.

On the contrary, seven point mutations were observed within the gene encoding GDP-mannose 3,5-epimerase (Fig. 2.16) and six of these mutations were associated only with the top three fruit AsA containing genotypes. These data strongly support the contention that fruit AsA is influenced by genetic polymorphism within the coding region of the gene for GDP-mannose 3,5-epimerase and suggest a potential marker for high fruit AsA.

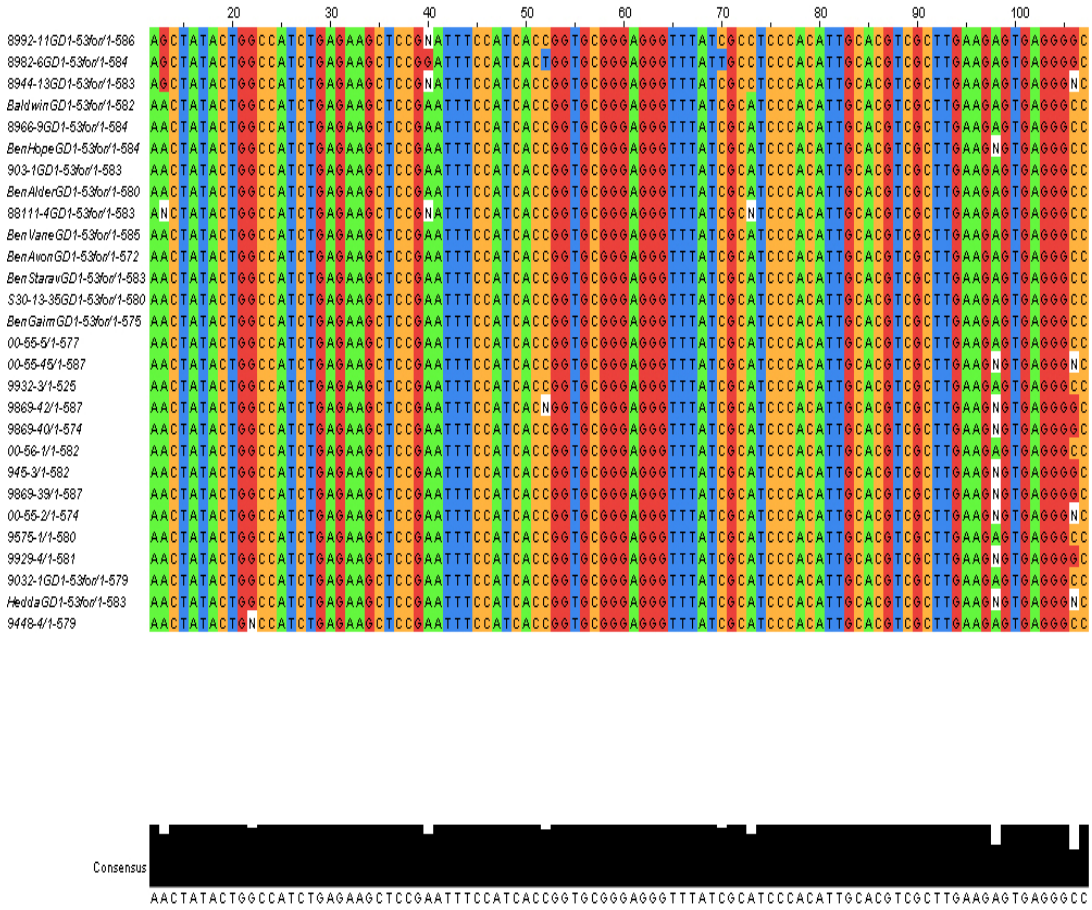


Figure 2.16 Alignment of a portion of the gene encoding GDP-mannose 3,5-epimerase from several blackcurrant genotypes

The GDP-mannose 3,5-epimerase encoding gene was cloned from cDNA of several blackcurrant genotypes containing a known fruit AsA concentration. Gene sequencing was performed and sequences aligned using the clustal program within Jalview. Genotype names are provided to the left of sequence and sequence are presented in descending order of fruit AsA concentration. Note the A → G change at position 13 for the highest three genotypes and the C → G change distributed throughout different genotypes at position 106.

Objective 3 – Define internal and external factors affecting berry AsA content

Rationale

Breeding novel high AsA blackcurrant cultivars is a long term project and can be expected to take in excess of 10 years even if advantage is taken of markers to allow accelerated breeding. In the short term, one objective of the project was to determine agronomic methods that may enhance the AsA concentration in blackcurrant fruit. In order to guide agronomic experiments, initial laboratory and field experiments were undertaken to determine which factors had the capacity to influence fruit AsA concentration.

Headlines

- There was a strong influence of environment on fruit AsA concentration with large temporal fluctuations
- All cultivars responded in a similar fashion to environmental variation and cultivar hierarchy was generally maintained year to year
- No single environmental variable had a major influence on fruit AsA concentration
- Irrigation had a positive impact on fruit yield in cv. Baldwin
- Reflective mulches positively influence fruit yields
- Manipulation of sink (flower removal) and source (leaf removal) impacted fruit yields but not AsA concentration
- Nitrogen nutrition had little impact on fruit yield but decreased fruit AsA concentrations

Analysis of historical records

A series of records regarding regional fruit AsA concentrations on a cultivar and annual basis were interrogated and compared with meteorological data in order to determine the influence of environment on fruit AsA concentration. Available records included (i) ascorbic acid data on a grower by grower basis of fruit delivered to the GSK factory at Coleford 1991-1996 and 2002-2003, (ii) cultivar assessment data from New Zealand trials 1989-1997 and (iii) data from UK cultivar assessments at SCRI (1972-2003), Brogdale (1972-1984), Luddington (1973-1984), Bradenham (1984-1992) and the West Midlands (1984-1992). Many of the data sets were incomplete or cultivars had only been grown at

particular locations over very short periods (2-3 years) which precluded detailed statistical analysis however, data regarding the AsA concentration of fruit from the cvs Baldwin, Ben Tirran, Ben Alder and Ben Lomond was sufficiently complete to allow correlations to be generated against various meteorological parameters.

Correlations were generated between fruit AsA concentration at harvest in any particular year and either total solar radiation, number of hours of unobscured sunshine, total rainfall or average temperature. They were generated for the time periods (i) twelve months preceding harvest, (ii) each individual month preceding harvest (e.g. is there an impact at the time of fruit AsA accumulation) and (iii) for each possible quarter prior to harvest (e.g. Sep-Nov, Oct-Dec, Nov-Jan etc.).

i. Year-on-year fruit AsA variation

Fig. 3.1 shows a plot of fruit AsA concentration of four different blackcurrant cultivars against year of harvest between the years 1972-2003. All four cultivars show a similar trend in AsA concentration with relatively high AsA in the mid 1970's declining to a minimum in fruit harvested around 1990 before rising again up towards the present day. Minimum fruit AsA concentrations were in the region of 100-150 mg/100 ml juice while maximum AsA concentrations were in the region of 250-300 mg/100 ml juice demonstrating the magnitude of environmental impact on fruit AsA concentrations. In general the fruit AsA concentration maintains the same hierarchy with respect to cultivar year on year. These data suggest that (i) environmental effects on fruit AsA concentration can be significant, (ii) environmental effects are independent of cultivar and all cultivars respond in the same way to the same environment and (iii) there is strong genetic over fruit AsA concentration with different cultivars maintaining the same hierarchy under different growing conditions.

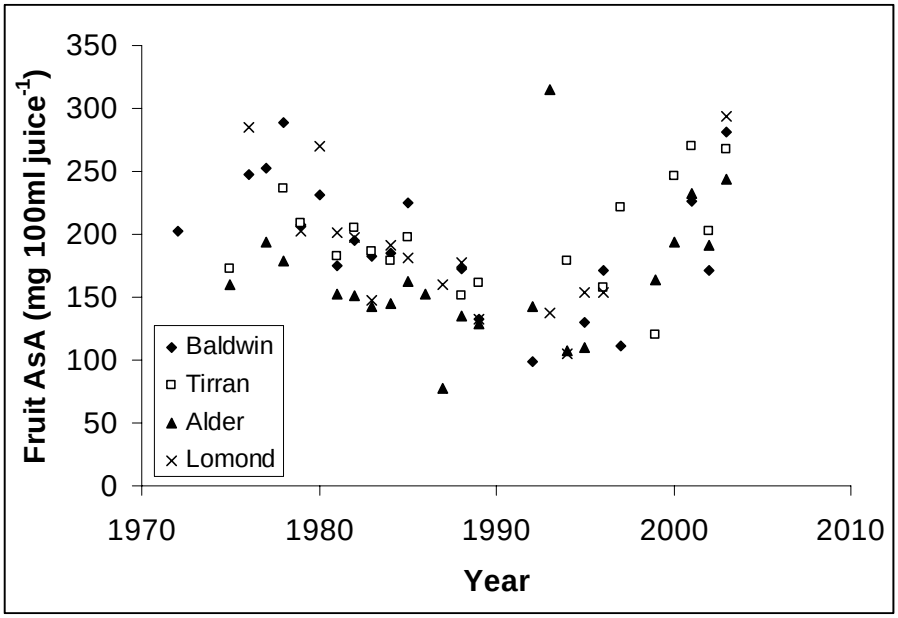


Figure 3.1 Variation of fruit AsA concentration of four blackcurrant cultivars harvested in different years at SCRI

Plants were grown as part of the ongoing SCRI breeding programme. Fruit were harvested at maturity, AsA extracted and determined by titration or HPLC.

ii. Impact of temperature

Previous work has demonstrated the potential impact of temperature on fruit vitamin C concentration. For example, elevated temperatures resulted in up to thirds reduction in AsA concentration in kiwifruit (Richardson *et al.*, 2004). Therefore the impact of temperature on fruit AsA concentration was determined. Table 3.1 shows the correlation coefficient (perfect correlation = 1) for correlations between average temperature within the time frame listed and fruit AsA concentration at harvest of the same year. In general, correlations were exceedingly low and the few cases where higher correlations were observed, there was no consistency between cultivars. For example, in Ben Lomond there was a relatively high correlation coefficient between average June temperature and fruit AsA concentration of fruit harvested in the same year (0.25), however in the other cultivars examined this correlation was exceeding low(< 0.03). Given the observation that all cultivars behaved in a similar fashion to the changing growing environment (Fig. 3.1), it is unlikely that the observed higher correlations result from anything other than chance.

Date	Cultivar			
	Baldwin	**Tirran**	**Alder**	**Lomond**
Sep-Aug	0.1580	0.0003	0.0114	0.0904
Sep	0.0271	0.1729	0.1027	0.0934
Oct	0.0050	0.0398	0.0114	0.0413
Nov	0.0196	0.0334	0.0005	0.0163
Dec	<0.0001	0.0242	0.0358	0.0299
Jan	0.0974	0.1078	0.0208	0.0470
Feb	0.2680	0.0378	0.0478	0.0052
Mar	0.0340	0.0187	0.1068	0.0062
Apr	0.0134	<0.0001	0.0077	0.1881
May	0.0025	0.0711	0.0481	0.0900
Jun	0.0271	0.0025	0.0100	0.2463
Jul	0.0284	0.0145	0.0599	0.0008
Aug	0.0578	0.0120	0.0087	0.0207
Sep-Nov	0.0081	0.1161	0.0023	0.0786
Oct-Dec	0.0004	0.0093	0.0318	0.0597
Nov-Jan	0.0108	0.0027	0.0011	0.0174
Dec-Feb	< 0.0001	0.0404	0.0354	0.0065
Jan-Mar	0.2084	0.0459	0.0798	0.0142
Feb-Apr	0.1661	0.0019	0.0790	0.0323
Mar-May	0.0337	0.0368	0.1083	0.0888
Apr-Jun	0.0258	0.0157	0.0365	0.3557
May-Jul	0.0358	0.0040	0.0006	0.1466
Jun-Aug	0.0636	0.0002	0.0120	0.0705
Jul-Sep	0.0280	0.0004	0.0187	0.0445
Aug-Oct	0.0001	0.1231	0.0381	0.1823

Table 3.1 Pearson correlation coefficients between fruit AsA concentration and average temperature within each pre-harvest time period for four blackcurrant cultivars

iii. Impact of rainfall

Reduced irrigation of leeks resulted in enhanced AsA levels (Sorensen *et al.*, 1995) and similarly, a correlation was observed between precipitation during head development and AsA content at harvest in broccoli (Toivonen *et al.*, 1994). Therefore correlations between total precipitation at different stages of fruit development and fruit AsA concentrations at harvest were investigated. As was observed with temperature, correlations were generally poor (Table 3.2).

Date	Cultivar			
	Baldwin	Tirran	Alder	Lomond
Sep-Aug	0.1524	0.0048	0.0712	0.0203
Sep	0.0364	<0.0001	0.0617	0.0152
Oct	0.0787	<0.0001	0.0193	0.0027
Nov	0.0164	0.0247	0.0411	0.0076
Dec	0.0171	0.0242	0.0274	0.0009
Jan	0.0735	0.0521	0.1029	0.0305
Feb	0.0294	0.0701	0.0023	0.0012
Mar	0.0036	0.0207	0.0711	0.0685
Apr	0.0478	0.0090	0.0768	0.2249
May	0.0038	0.0027	0.0680	0.0239
Jun	0.0022	0.0034	0.0222	0.0023
Jul	0.0117	0.0020	0.0022	0.0543
Aug	0.0140	0.0069	0.0004	0.0286
Sep-Nov	0.0911	0.0055	0.1063	0.0063
Oct-Dec	0.0952	0.0477	0.0225	0.0015
Nov-Jan	0.0384	0.0170	0.0109	0.0178
Dec-Feb	0.0358	0.0600	0.0505	<0.0001
Jan-Mar	0.0483	0.0030	0.0003	<0.0001
Feb-Apr	0.0510	0.0038	0.0073	0.0303
Mar-May	0.0130	0.0255	0.0138	0.0007
Apr-Jun	0.0383	0.0028	0.0349	0.0351
May-Jul	0.0142	0.0011	0.0099	0.0374
Jun-Aug	<0.0001	0.0015	0.0018	<0.0001
Jul-Sep	0.0040	0.0021	0.0102	0.0125
Aug-Oct	0.1379	0.0001	0.2483	0.0060

Table 3.2 Pearson correlation coefficients between fruit AsA concentration and total precipitation within each pre-harvest time period for four blackcurrant cultivars

iv. Impact of solar radiation

In photosynthetic tissues, several groups have demonstrated an interaction between AsA synthesis and light intensity (Gatzek *et al.*, 2002; Bartoli *et al.*, 2006; Dowdle *et al.*, in press) and exposure to light was demonstrated to impact AsA concentration in apple fruit (Davey *et al.*, 2004). Therefore correlations between total solar radiation, number of hours of unobscured sunlight and fruit AsA concentrations were performed.

Low correlations were observed between fruit AsA concentration and total sunlight hours in the four cultivars (table 3.3).

	Cultivar			
Date	**Baldwin**	**Tirran**	**Alder**	**Lomond**
Sep-Aug	0.0424	0.0016	0.1682	0.0054
Sep	0.2023	0.0277	0.1929	<0.0001
Oct	0.0034	0.0377	0.0469	0.0708
Nov	0.0007	0.0117	0.0262	<0.0001
Dec	0.0019	0.0502	0.0952	0.0060
Jan	0.0228	0.1203	0.0141	0.0027
Feb	0.0847	0.0016	0.0002	0.0193
Mar	0.0169	0.0288	0.0145	0.0163
Apr	0.1009	0.1308	0.0002	0.2399
May	0.0158	0.0195	0.0170	0.0028
Jun	0.0016	0.0027	0.0553	0.0152
Jul	0.0405	0.1109	0.0342	0.0160
Aug	0.0155	0.0449	0.0770	0.0052
Sep-Nov	0.1332	0.0277	0.1360	0.0255
Oct-Dec	0.0005	0.0286	0.0406	0.0424
Nov-Jan	0.0135	0.0011	0.0023	0.0140
Dec-Feb	0.0119	0.0114	0.0677	0.0579
Jan-Mar	0.0206	0.0589	0.0138	0.0195
Feb-Apr	<0.0001	0.0891	0.0047	0.0224
Mar-May	0.0002	0.0943	0.0223	0.0177
Apr-Jun	0.0071	0.0429	0.0414	0.0114
May-Jul	0.0332	0.0167	0.0726	0.0242
Jun-Aug	0.0212	0.106	0.1030	0.0087
Jul-Sep	0.0743	0.1231	0.1111	0.0342
Aug-Oct	0.0414	0.0502	0.1845	0.0813

Table 3.3 Pearson correlation coefficients between fruit AsA concentration and total sunlight hours within each pre-harvest time period for four blackcurrant cultivars

Similar results were obtained when total solar radiation in each of the time periods pre-harvest was correlated with fruit AsA concentration. Generally low correlation coefficients were observed with little consistency between cultivars (table 3.4).

Date	Cultivar			
	Baldwin	**Tirran**	**Alder**	**Lomond**
Sep-Aug	0.2001	0.1513	0.0616	0.2175
Sep	0.0608	0.0473	0.0672	0.0981
Oct	0.1392	0.0636	0.0361	0.0399
Nov	0.0428	0.1114	0.1525	0.1306
Dec	0.0005	0.0006	0.0146	0.0032
Jan	0.0782	0.4078	0.0673	0.0604
Feb	0.0022	0.0236	0.0538	0.0004
Mar	0.0037	0.1501	0.0297	0.0008
Apr	0.0479	0.2138	0.0268	0.3297
May	0.0553	0.0157	0.0121	0.0227
Jun	0.0019	0.0015	0.0214	0.0261
Jul	0.0759	0.0459	0.0331	0.0075
Aug	0.1560	0.0552	0.0368	0.1820
Sep-Nov	0.0033	0.0070	0.0090	0.1777
Oct-Dec	0.1192	0.0853	0.0960	0.1007
Nov-Jan	0.0836	0.0675	0.1832	0.1124
Dec-Feb	0.0078	0.1440	0.1920	0.0002
Jan-Mar	<0.0001	0.2251	0.0683	0.0050
Feb-Apr	0.0131	0.2452	0.0547	0.2082
Mar-May	0.0616	0.1365	0.0311	0.1332
Apr-Jun	0.0605	0.0778	0.0291	0.1644
May-Jul	0.1389	0.0439	0.0513	0.0435
Jun-Aug	0.1748	0.0979	0.0637	0.0898
Jul-Sep	0.1657	0.2707	0.0445	0.0637
Aug-Oct	0.0372	0.1985	0.0059	0.0241

Table 3.4 Pearson correlation coefficients between fruit AsA concentration and total solar radiation within each pre-harvest time period for four blackcurrant cultivars

v. Conclusions

While there is clearly an environmental impact on fruit AsA concentration (Fig. 3.1), no correlations were observed between single environmental variables and fruit AsA at harvest in the following season (tables 3.1-3.4). These data suggest a complex interaction between plant genetics and the environment with overlapping impacts between environmental variables.

Impact of aspect on fruit AsA concentration

As part of ongoing efforts to determine the impact of light on fruit AsA concentration, fruit were harvested from five separate bushes growing on a North or South facing slope at the same location on a commercial plantation close to Dundee. A significant difference ($P < 0.001$) was observed in fruit AsA concentration between those bushes grown on the South

facing slope which had almost 25% higher AsA concentrations than those grown on the North facing slope (2.03 ± 0.08 and 1.64 ± 0.14 mg gFW^{-1}, mean ± SE respectively). These data suggest a possible cumulative impact of receiving greater solar radiation year on year.

Effect of temperature on AsA production

In order to measure the impact of temperature on fruit AsA accumulation in the absence of other environmental variables, plants were transferred to controlled environment cabinets immediately after bud burst and allowed to develop to maturity as described in materials and methods. There was a trend to higher fruit AsA concentration at lower temperatures. In both Hedda and Baldwin cvs day/night temperatures of 15°C/10°C were optimum for AsA accumulation (Fig. 3.2).

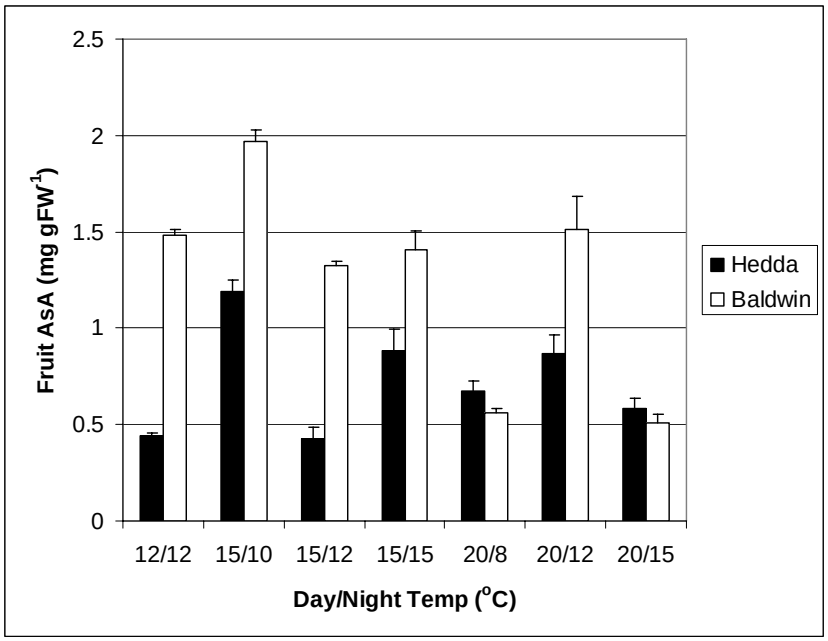


Figure 3.2 The effect of growth temperature on fruit AsA content in blackcurrant

Plants were transferred to controlled environment chambers prior to bud burst and maintained under the temperature regime shown until fruit reached maturity. Values shown are mean ± SE of fruit AsA concentration (n = 5).

Manipulation of water supply

Year 2 (2003)

Effects of differences in irrigation on fruit development and [AsA] in potted bushes

Imposition of partial root drying did not significantly affect fruit yield, nor the proportion of ripe fruit or the proportion of fruit in the different size categories ($P > 0.1$ for yield and proportions). However, there was a significant treatment effect on fruit development prior to harvest, when fruit sizes on tagged strigs were measured on 14 May 2003 ($P = 0.046$). The mean diameters of fruit for the early PRI treatment were smaller than those for the other treatments, for both Baldwin and Hedda (4.8 mm for early PRI, compared to 5.8 mm, 5.6 mm and 5.6 mm for continuous PRD, late PRI and control, respectively, averaged over both cultivars). These differences were, however, no longer apparent closer to final harvest (12 June 2003; $P = 0.42$).

When comparing dry matter allocation, cultivar and treatment differences were apparent (Fig. 3.3). There was a significant cultivar effect on the percentage allocation of dry matter to the leaves, new shoots, old shoots and roots ($P \leq 0.004$), with Baldwin showing a greater allocation of dry matter to leaves, new shoots and roots than Hedda. These results show that the growth of the cv. Baldwin was more vigorous as it allocated more of its dry matter, proportionally, to the new season's growth. Interestingly, the proportions of dry matter allocated to the fruits were similar for both cultivars ($P = 0.37$). The control treatments for both cultivars appeared to show a slightly greater allocation of dry matter to leaves, and a slightly lower allocation of dry matter to roots, than the PRI treatments, although treatment effects were not significant at the 5% level ($P = 0.09$ and 0.07, respectively). There was no evidence to suggest that any of the PRD treatments had a major impact on reducing vegetative growth and leaf area production, nor was there any impact on root growth. The way and extent to which the PRI treatments were applied will require further attention if it is going to be of practical use. Interestingly, there was a treatment effect of PRI on berry [AsA] only in cv. Baldwin. Table 3.5 below shows the berry [AsA] values for ripe berries.

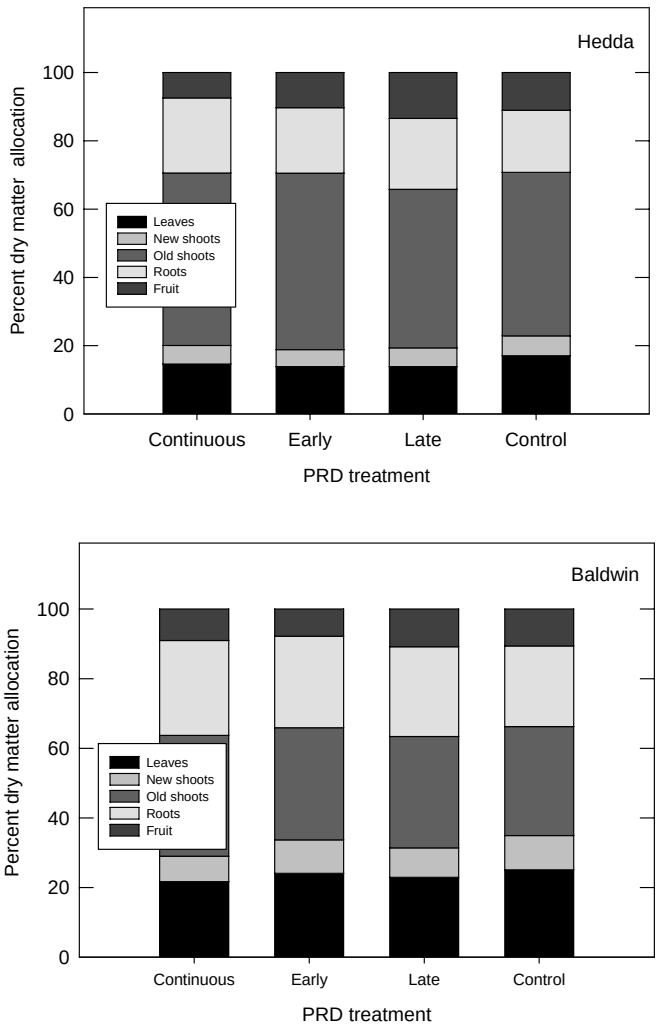


Figure 3.3 The effects of partial root drying (PRD) on the cvs Hedda (left) Baldwin (right), applied over three different periods of time, on the allocation of dry matter to leaves, old and new shoots, roots and fruits

[AsA] in Baldwin berries was approximately twice that in Hedda berries, as noted for the nitrogen and crop load experiments. There was no significant PRI treatment effect on berry [AsA] for the medium-sized berries (diameter 8-11.2 mm). However, there was a significant cultivar × PRI treatment interaction for [AsA] in the larger berries ($P = 0.04$), suggesting that the two cultivars responded differently to PRI. Comparison of treatment mean values for Baldwin showed that there was a significantly higher concentration in berries from the early PRI treatment compared to control (no PRI) or later PRI. Continuous PRD yielded berries with [AsA] significantly higher than late PRD. Since the

above significant treatment effects were confined to the large berries for Baldwin, a further set of Baldwin berries were extracted for confirmation. The results are shown in Table 3.5.

Table 3.5 The effects of partial root irrigation (PRI) on fruit total [AsA] for cvs Hedda and Baldwin. Fruit of two different size classes (8 to 11.2 mm and >11.2mm) were analysed

	[AsA] (mg g^{-1}) (ripe berries 8-11.2 mm)		**[AsA] (mg g^{-1}) (ripe berries > 11.2 mm)**	
Cultivar	*Baldwin*	*Hedda*	*Baldwin*	*Hedda*
Treatment				
Control (no PRI)	2.22	1.25	1.86	1.04
Early PRI	2.09	1.20	2.03	1.00
Late PRI	2.19	1.21	1.77	1.04
Continuous PRI	2.14	1.22	1.92	1.05
P value Cultivar		*<0.001*		*<0.001*
PRI		*0.62*		*0.13*
Cultivar x PRI		*0.88*		*0.04*
*LSD (29 d.f.) **		*0.21*		*0.14*

Note: LSD values quoted are for comparisons between treatments within a specific cultivar.

Table 3.6 The effects of partial root irrigation (PRI) on fruit total [AsA] for cv. Baldwin. The second analysis of fruit (>11.2mm)

Treatment	**[AsA] (mg g^{-1}) in ripe Baldwin berries >11.2 mm**
Control	1.79
Early PRI	1.96
Late PRI	1.79
Continuous PRI	1.76
LSD (30 d.f.)	*0.13*

Note: LSD values quoted are for comparisons between treatments within a specific cultivar.

This second set of results confirms that early PRI treatment significantly elevated [AsA] in large (>11.2 mm) berries in Baldwin, but suggests that continuous PRI did not have a significant effect (Table 3.6).

Year 3 (2004)

Effects of differences in irrigation on fruit development and [AsA] in field-grown bushes

Soil, under the mulch, that was not irrigated quickly dried out, and reached a water potential of between -300 and -400 HPa by mid May and remained at this water potential until harvest (Fig. 3.4). A similar pattern was exhibited in soil which was not covered by plastic mulch and did not receive irrigation. Soil receiving full irrigation (i.e. on both sides of the bush) was kept close to field capacity until after harvest, never falling below -100 HPa. Those bushes receiving irrigation on one side of the bush, had dry soil on the non-irrigated side (PRI East) and soil close to field capacity on the side receiving irrigation (PRI West), when the irrigation was switched over on the 28 May, the previously dry side had re-hydrated to a moisture level similar to fully irrigated soil by the 4 June and the previously irrigated soil gradually dried until reaching the maximum deficit by 15 June 2004.

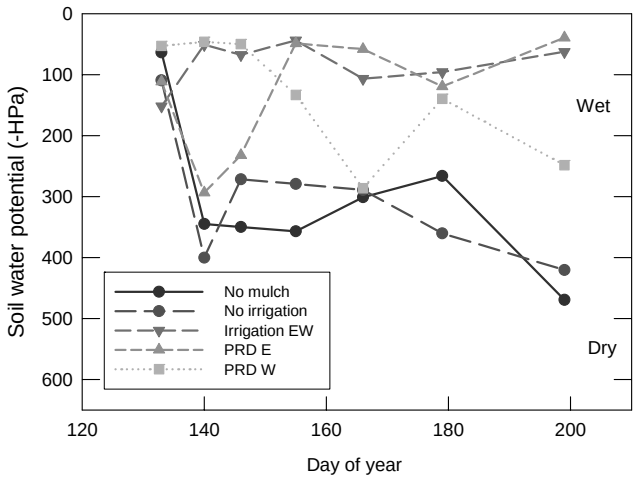


Figure 3.4 The effect of different methods of irrigation, including partial irrigation, on soil water potential

Full or partial irrigation had a significant effect on the growth of the bushes. Mean shoot length and total shoot length were increased by both irrigation treatments (Table 3.7). Total shoot lengths per bush were 41% and 35% greater for those receiving full and partial irrigation respectively than those not receiving irrigation.

Irrigation had no effect on the total harvest yield per bush, but influenced the proportion of berries within each size class (Table 3.8). Bushes receiving partial root drying (PRI) and full irrigation had a higher proportion of berries in the largest size class (>11.2 mm diameter). This was reflected by the mean berry weight which was greater for bushes in either of the irrigation treatments compared to those in the non-irrigated treatment.

Table 3.7 The effect of different methods of irrigation regimes on Baldwin bush vegetative shoot growth

Treatment	Number of shoots	Total shoot length (cm)	Average shoot length (cm)
None	23	892	39
PRI	24	1200	50
Full	25	1257	50
SED (12 d.f.)	*1.5*	*64.2*	*1.9*
P	*0.229*	*<0.001*	*<0.001*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.8 The effects of different methods of irrigation on total crop per Baldwin bush and the proportion of berries in different size classes

Treatment	Fruit size class (diameter, mm)						Mean berry wt (mg)	Total	
	>11.2		8-11.2		5-8				
	Yield (g)	%	Yield (g)	%	Yield (g)	%		No of berries	Yield (g)
None	441	40	637	58	26	2	717	1543	1105
PRI	540	47	596	52	19	2	771	1502	1155
Full	572	47	620	51	22	2	789	1562	1215
SED (12 d.f.)	*65.8*		*51.0*		*2.37*		*218.1*	*116.9*	*103.6*
P	*0.161*	*0.012*	*0.727*	*0.013*	*0.047*	*0.068*	*0.016*	*0.872*	*0.584*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

The concentration of AsA was greatest in the berries within size class 8-11.2 mm diameter and similar for the larger (>11.2 mm) and smaller (5-8 mm) berries (Table 3.9). The different methods of irrigation did not influence concentration of AsA for the berries >8 mm diameter, but PRI reduced the concentration compared to full irrigation or no irrigation for the smallest berries (5-8 mm diameter). The method of irrigation had no effect on the total AsA content of the bushes (Table 3.10) although the total content of AsA in the berries >11.2 mm was increased for bushes receiving either full irrigation or PRI.

Table 3.9 The effects of different methods of irrigation on Baldwin berry AsA concentration (mg g^{-1} fresh weight)

Treatment	Fruit size class (diameter, mm)		
	>11.2	**8-11.2**	**5-8**
None	2.19	2.53	2.14
PRI	2.14	2.55	1.93
Full	2.21	2.54	2.09
SED (12 d.f.)	*0.048*	*0.054*	*0.069*
P	*0.325*	*0.900*	*0.026*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.10 The effects of different methods of irrigation on total Baldwin bush AsA content (mg)

Treatment	Fruit size class (diameter, mm)			Total
	>11.2	**8-11.2**	**5-8**	
None	953	1600	56	2610
PRI	1156	1535	37	2729
Full	1265	1575	47	2887
SED (12 d.f.)	*154.3*	*148.6*	*5.7*	*268.9*
P	*0.165*	*0.907*	*0.023*	*0.599*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Year 4 (2005)

Effects of differences in irrigation on fruit development and [AsA] in field-grown bushes

Currently, there is little evidence that on East Malling soils (free draining on ragstone) that the treatments imposed any measurable differences in stress (leaf water potentials) to the bushes. Measurements of leaf water potential do however show varietal differences, with the larger older Baldwin having the more negative values and therefore the greater stress levels than the younger Ben Lomond (Table 3.11). Drought stress levels were always greater with Baldwin irrespective of date and for the no irrigation treatment but the differences were small.

Measurements were made of soil moisture content using *ThetaProbes* (Delta-T Devices) buried in the soil at fixed positions and a constant depth. For the treatment where water was only applied to half the root system, both sides on each bush were measured, i.e. east and west (Fig. 3.5). Soil moisture content was generally always highest for the full treatment and indicative of soil that was generally well-irrigated. While, with the other

treatments the soil was drier and the switch of the irrigation system from the west to the east side was clearly picked up by the *ThetaProbes*.

Table 3.11 Mean xylem leaf water potentials (-MPa) of the cvs Ben Lomond and Baldwin when subjected to various irrigation treatments throughout the growing season and sampled during the growing season of 2005

Date	Day of the year	Treatment	Ben Lomond	Baldwin
23 May	143	No irrigation	9.03	11.86
		Partial irrigation	8.00	12.43
		Full irrigation	8.56	11.12
9 June	160	No irrigation	8.68	12.34
		Partial irrigation	7.73	12.31
		Full irrigation	8.06	12.84
22 June	173	No irrigation	7.68	9.72
		Partial irrigation	7.11	9.04
		Full irrigation	7.06	9.34

Despite the lack on any measurable influence of the different irrigation treatments on plant leaf water potentials there is some evidence to suggest that yield of Baldwin may have been positively influenced by irrigation (Fig. 3.6). This was not, however, the case with the younger, smaller Ben Lomond.

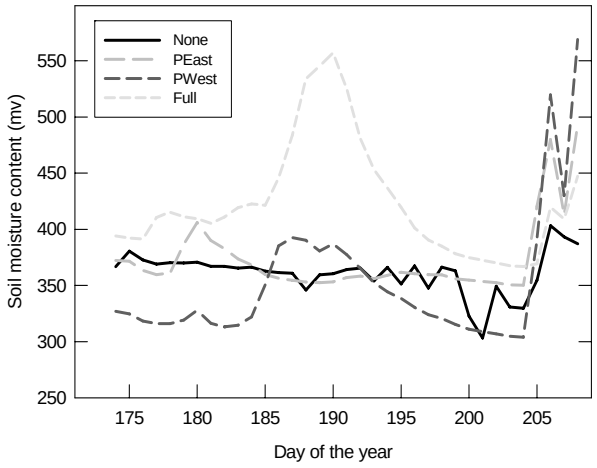


Figure 3.5 Mean field soil moisture content (mv) determined from *in situ ThetaProbe* (Delta-T Devices) measurements

Note: Treatments are as follows: None, no applied irrigation; PEast, watering on one side of the bush; PWest watering on the other side of the bush and Full, irrigation on both sides. Irrigation when applied was applied at the same rate irrespectively of dripper location.

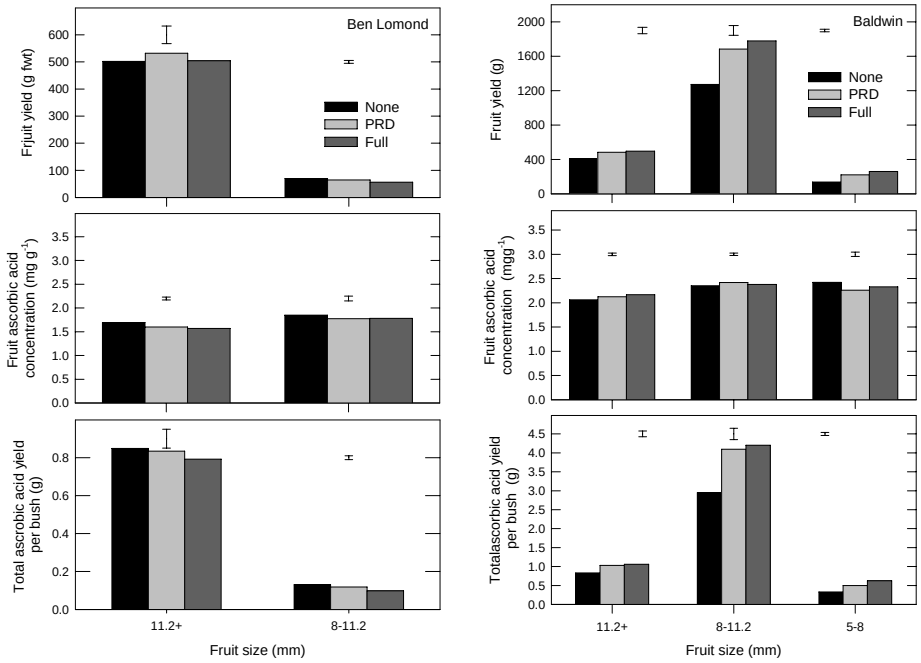


Figure 3.6 Fruit yields (g per fresh weight per bush), [AsA]s (mg g^{-1}) and total amount of ascorbic acid (g per bush) of the cvs Ben Lomond (left) and Baldwin (right) when grown under different trickle irrigation regimes

Measurements of fruit [AsA]s were very consistent within a cultivar and also suggest that none of the irrigation treatments have had any impact (Fig. 3.6). Differences in the yield of ascorbic acid per bush ([AsA] × fruit yield) were due only to differences in fruit yield.

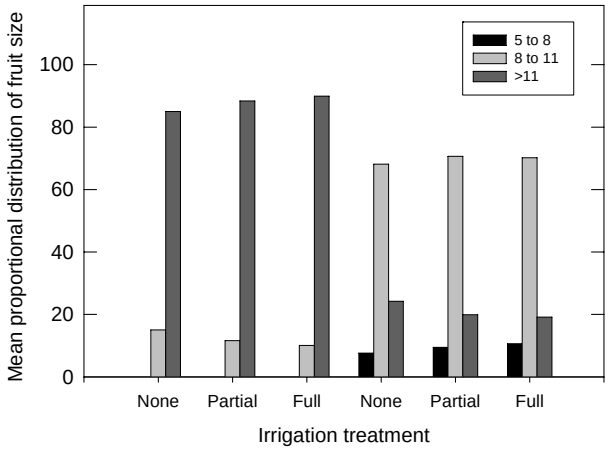


Figure 3.7 Mean proportional distribution of fruit size to three categories (5.6 to 8.2 mm, 8.3 to 11.2mm and >11.2mm) for the cvs Ben Lomond and Baldwin when grown under three different irrigation regimes

There was little evidence to suggest that the positive impact of irrigation on yield was due to a dramatic increase in berry (quality) size (Fig. 3.7). This suggests that irrigation enhanced yield by increasing fruit number not size.

Manipulation of light interception

Year 3 (2004)

Effects of enhanced solar radiation interception using reflective mulches

'Extenday' was the most efficient at returning light hitting the ground back into the canopy, an average of 37% of the incoming light was reflected, compared with 19% for 'Solarmat' and 9% for bare earth treatment (Fig. 3.8). The reflective mulches did not have any noticeable effects on the air temperature at 15 cm or 30 cm within the bush canopy (Fig. 3.8). Both of the reflective mulch treatments reduced mean daily soil temperatures compared to bare soil (Fig. 3.8). This confirmed that the reflective mulches increase the amount of PAR reflected from ground level.

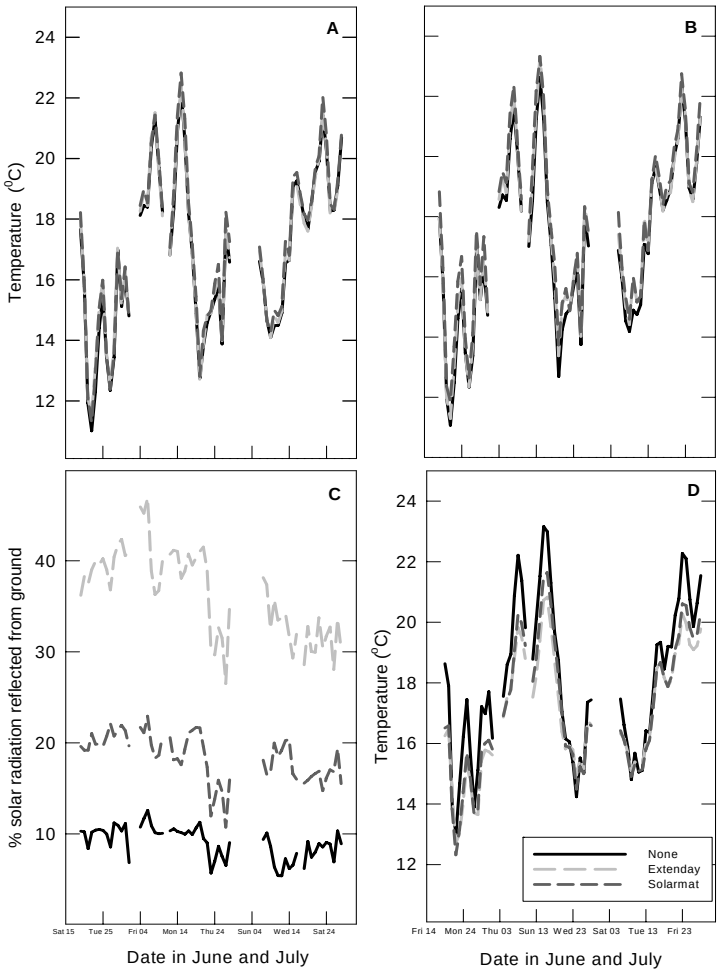


Figure 3.8 The effect of reflective mulches on air temperature and PAR around Baldwin bushes. A) air temperature in the bush canopy 15cm from ground B) air temperature in the bush canopy 30cm from ground C) % incoming radiation (PAR) reflected back, and D) soil temperature at 10cm depth

The reflective mulches increased the growth of the bushes, mostly likely due to the increased radiation intercepted by the bush leaf canopy. Total bush shoot length was increased by 'Extenday' and 'Solarmat' by 30% and 16% respectively when compared to the bare soil treatment. This was due to an increase in the average shoot length; there was no increase in the number of new shoots (Table 3.12). Chlorophyll content of the leaves between May and July (Fig. 3.9) was increased by the reflective mulch treatments.

Total harvest yield was 4% greater for bushes planted into 'Extenday' and 11% greater for those in 'Solarmat' than for those in bare soil (Table 3.13). However, these effects were not statistically significant. The reflective mulches did not have consistent or significant effects on the proportion of berries within different size classes (Table 3.13).

[AsA] was greatest for the berries within the 8-11.2 mm size class (Table 3.14). For berries >8 mm diameter on the bushes in either of the reflective mulches, AsA concentration was greater than for those on bushes in bare soil (Table 3.14). The total AsA content of the bushes was increased by 5% with 'Extenday' and 16% by 'Solarmat' compared to bare soil, but neither of these effects were statistically significant (Table 3.15).

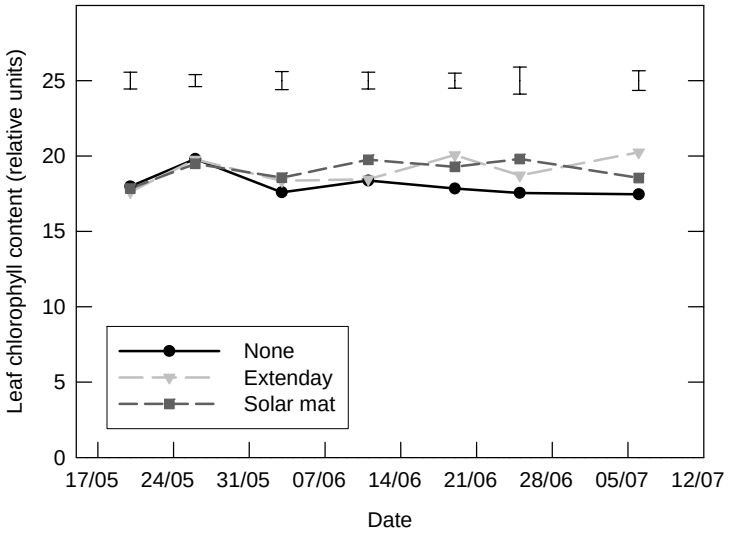


Figure 3.9 The effect of reflective mulches on the 'chlorophyll content' of extension leaves of Baldwin bushes during the growing season

Table 3.12 The effect of reflective mulch on Baldwin bush vegetative shoot growth

Treatment	No. of shoots	Total shoot length(cm)	Mean shoot length (cm)
No mulch	25	780	30.7
'Extenday'	28	1005	37.2
'Solarmat'	26	908	35.9
SED (12 d.f.)	*2.0*	*72.7*	*0.17*
P	*0.559*	*0.024*	*0.006*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.13 The effects of reflective mulches on total crop per Baldwin bush and the proportion of berries in different size classes

Treatment	Fruit size class (diameter, mm)						Total	
	>11.2		8-11.2		5-8			
	Yield (g)	%	Yield (g)	%	Yield (g)	%	No. of berries	Yield (g)
No mulch	363	39	569	59	25	3	1358	957
'Extenday'	401	40	568	57	24	3	1352	993
'Solarmat'	362	35	665	62	32	3	1519	1059
SED (12 d.f.)	*37.4*		*71.6*		*4.8*		*169.7*	*88.5*
P	*0.513*	*0.266*	*0.332*	*0.268*	*0.302*	*0.371*	*0.553*	*0.524*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.14 The effects of reflective mulch on Baldwin berry AsA concentration (mg g^{-1} fresh weight)

Treatment	Fruit size class (diameter, mm)		
	>11.2	8-11.2	5-8
No mulch	2.32	2.77	2.22
'Extenday'	2.40	2.80	2.89
'Solarmat'	2.43	2.89	2.17
SED (12 d.f.)	*0.047*	*0.054*	*0.064*
P	*0.094*	*0.105*	*0.753*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.15 The effects of reflective mulch on total AsA content of the Baldwin bush (mg)

Treatment	Fruit size class (diameter, mm)			Total
	>11.2	8-11.2	5-8	
No mulch	842	1568	55	2465
'Extenday'	957	1577	52	2586
'Solarmat'	881	1909	69	2859
SED (12 d.f.)	*87.6*	*199.4*	*9.8*	*229.6*
P	*0.434*	*0.192*	*0.249*	*0.253*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

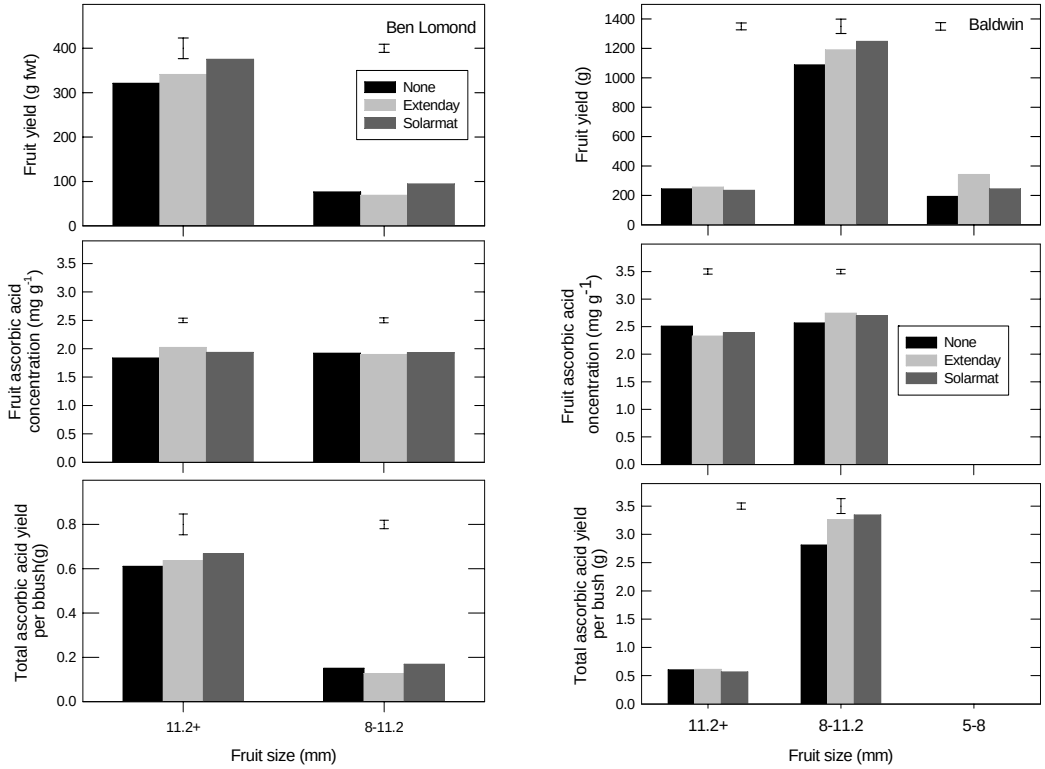


Figure 3.10 Fruit yields (g per fresh weight per bush), [AsA] (mg g^{-1}) and total amount of ascorbic acid (g $bush^{-1}$) of the cvs Ben Lomond (left) and Baldwin (right) when grown for an entire season with light reflective soil mulches

There was some evidence to suggest that the reflective mulch treatments positively influenced fruit yield per bush (Fig. 3.10). This was particularly true for both reflective materials and the cv. Baldwin (around 15% increase), whereas only the Solarmat appeared to increase the yield of Ben Lomond.

Measurements of fruit [AsA] were very consistent within a cultivar and also suggest that none of the reflective mulches had any influence (Fig. 3.10). Differences in the yield of ascorbic acid per bush ([AsA] x fruit yield) were due only to differences in fruit yield (Fig. 3.10). Analysis of the proportional size distribution of fruit to various size classes suggests that the reflective mulches had no influence on fruit size for either Ben Lomond or Baldwin (Fig. 3.11).

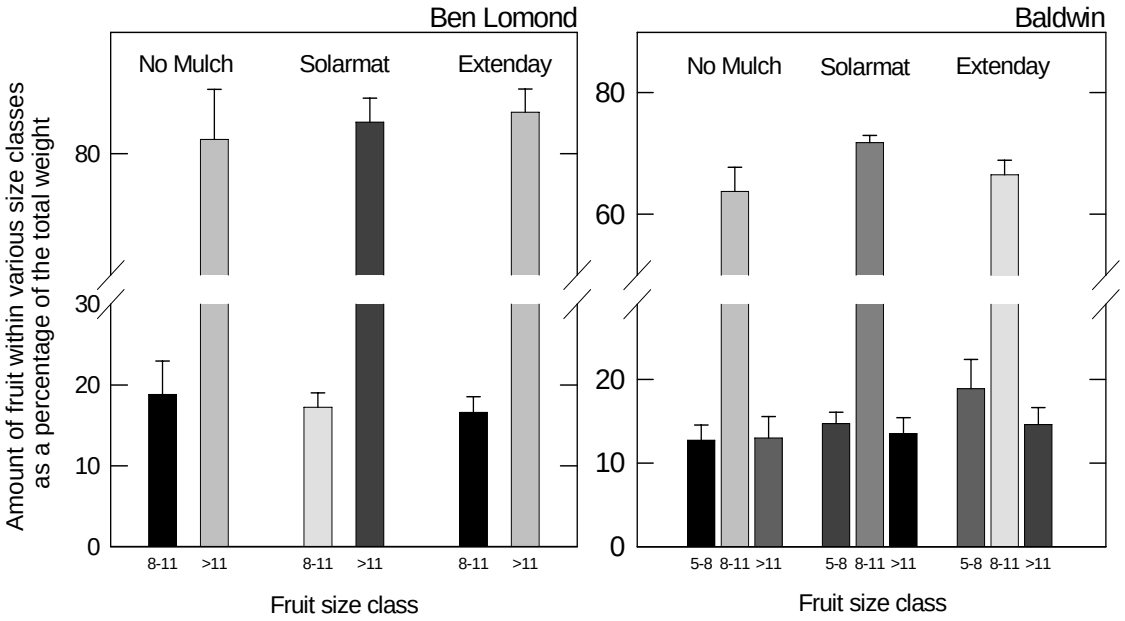


Figure 3.11 Proportional distribution of fruit to various size classes based on mean total fruit fresh weight per bush for Ben Lomond (left) and Baldwin (right) for an entire season with light reflective soil mulches

Seasonal measurements of chlorophyll content for leaves Ben Lomond and Baldwin are shown in Fig. 3.12. These data show clear seasonal changes in leaf chlorophyll content, different varietal patterns and total levels, but no obvious treatment influences.

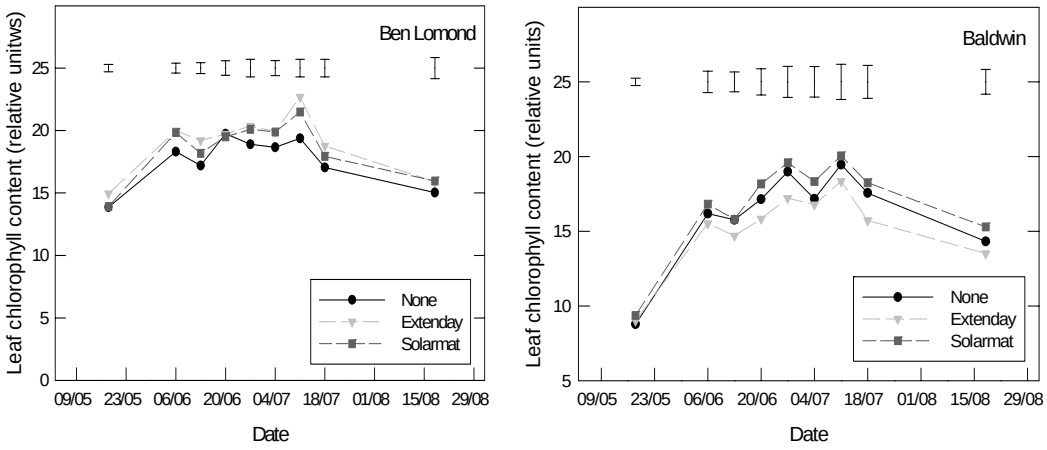


Figure 3.12 Mean seasonal measurements of chlorophyll content for leaves of the cvs Ben Lomond (left) and Baldwin (right) when grown for an entire season with light reflective soil mulches

Manipulation of crop load

Year 2 (2003)

Effects of strig removal on fruit development and [AsA]

The different levels of crop thinning clearly affected final crop yield (Table 3.16), but there was no significant treatment effect on the proportion of ripe fruit, nor on the proportion of fruit within the different size categories (data not shown).

Table 3.16 The effects of different levels of flower strig thinning (complete 'strig' removal) on total crop yield (g) and yield normalised to the control treatment

Cultivar	***Hedda***		***Baldwin***		**Mean**	
Thinning Treatment	Total crop yield (g)	Normalised yield (%)	Total crop yield (g)	Normalised yield (%)	Total crop yield (g)	Normalised yield (%)
Control (0%)	204	100	152	100	178	100
20%	146	72	139	91	142	80
50%	93	46	85	56	89	50
80%	56	27	70	46	63	35
P value Cultivar:					*0.48*	
Thinning:					*<0.001*	
Cultivar × thinning:					*0.58*	

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

There was no significant thinning treatment effect on [AsA] for large (diameter > 11.2 mm) or medium (diameter 8-11.2 mm) berries (P = 0.49 and 0.47, respectively). Baldwin berries had approximately twice the concentration of AsA as Hedda berries: total [AsA] of 1.91 and 1.79 mg g^{-1} for ripe Baldwin berries of diameter 8-11.2 mm and >11.2 mm, respectively, and total [AsA] of 1.19 and 0.78 mg g^{-1} for ripe Hedda berries of diameter 8-11.2 mm and >11.2 mm, respectively.

Year 3 (2004)

Effects of strig removal on fruit development and [AsA]

Reducing the fruit load by removal of 50% of each strig (thinning) increased total shoot extension, but had no effect on the number of new shoots. Thus, the average shoot length

was significantly increased (Table 3.17). This increased vegetative growth is a common response in fruit plants when crop load is reduced, since resources (carbohydrates) can be diverted from the fruit to the shoots.

Table 3.17 The effect removing 50% (T50) of the fruit from 'of every strig on vegetative shoot growth of Baldwin' bushes

Treatment	Number of shoots	Mean shoot length (cm)	Total shoot length (cm)
C	24	3.44	828
T50	23	3.85	872
SED (11 d.f.)	*2.0*	*0.16*	*8.2*
P	*0.527*	*0.028*	*0.608*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

The total harvest yield per bush was reduced by thinning (Table 3.18). Although the strigs had been thinned by 50%, the thinned bushes had only 22% less yield than the control bushes. At harvest, if all the berries remained on the strigs that had not been thinned, then the total number of berries per bush on the thinned bushes would have been 50% of the non-thinned. However, the total number of berries was 62% of the control (Table 3.18).

Table 3.18 The effect of removing 50% (T50) of each strig on the total crop per bush and the proportion of berries in different size classes from Baldwin bushes

Treatment	Fruit size class (diameter, mm)						Mean berry weight	Total	
	>11.2		8-11.2		5-8				
	Yield (g)	%	Yield (g)	%	Yield (g)	%	(mg)	No. of berries	Yield (g)
C	316	35	537	63	20	2	696	1340	874
T50	356	52	312	47	10	1.5	815	827	679
SED (11 d.f.)	*66.5*		*51.6*		*3.0*		*54.0*	*182.0*	*102.4*
P	*0.559*	*0.001*	*0.001*	*<0.001*	*0.007*	*0.026*	*0.050*	*0.017*	*0.083*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

This could be due to a greater loss of berries from natural drop prior to harvest on the control bushes, or lack of development of the terminal berries on the strigs. The thinned bushes compensated for loss of fruit during the season by significantly increasing mean berry size (Table 3.18) primarily by increasing the proportion of large berries (>11.2 mm diameter). AsA concentration was greatest in the berries 8-11.2 mm diameter and similar in the berries >11.2 and <8 mm diameter (Table 3.19). Thinning had no effect on the concentration of AsA in the remaining largest berries (i.e. >11.2 mm diameter) and caused a small decrease in the 8-11.2 mm diameter berries. However, in the smaller fruit (<8 mm) the concentration of AsA was significantly increased by thinning (Table 3.19). The total amount of AsA within the bush was reduced by 30% by thinning (Table 3.20). This was due to a large decrease in the total weight of AsA in the berries within the size class 8-11.2 mm.

Table 3.19 The effects of removing 50% (T50) of the fruit from each strig on berry AsA concentration (mg g^{-1} fresh weight) of Baldwin bushes

Treatment	Fruit size class (diameter, mm)		
	>11.2	**8-11.2**	**5-8**
C	2.66	3.36	2.57
T50	2.54	3.11	2.90
SED (11 d.f.)	*0.129*	*0.1319*	*0.149*
P	*0.383*	*0.086*	*0.051*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.20 The effect of removing 50% of the fruit from each strig on total bush AsA content (mg) of Baldwin bushes

Treatment	Fruit size class (diameter, mm)			Total
	>11.2	**8-11.2**	**5-8**	
C	835	1778	51	2660
T50	900	967	31	1930
SED (11 d.f.)	*206.0*	*173.5*	*7.91*	*327.0*
P	*0.758*	*<0.001*	*0.026*	*0.047*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Year 4 (2005)

As would be expected from the different ages (size) of the bushes of the two cultivars, there were marked differences in mean total fruit yield (Table 3.21, Fig. 3.13). The older Baldwin bushes yielded four times as much fruit as the younger Ben Lomond. When subject to early flower thinning, at a level designed to remove 50% of the flowers, there was as expected a reduction in total mean yield per bush. For Ben Lomond the mean yield reduction was slightly less than 50% of the unthinned mean yield per bush. The fact that it was not 50% suggests that there was some yield compensation, either by an increase fruit size, or an increase in the number of fruit retained. For Baldwin, however, there was little reduction in mean total yield with thinning (Table 3.21). This suggests that the ability of Baldwin bushes to compensate for the early flower thinning was greater. This may be due to differences in several factors such as, variety, bush size, bush age.

Table 3.21 The influence of early flower thinning on mean total fruit yield (g) per bush for the cvs Ben Lomond and Baldwin

Cultivar	Treatment	Yield (g) bush^{-1}
Ben Lomond	No thinning	335 $\pm$ 30
	Thinned by 50%	186 $\pm$ 29
Baldwin	No thinning	1326 $\pm$ 117
	Thinned by 50%	1230 $\pm$ 135

Despite large differences in fruit yield, particularly with Ben Lomond and the increase in fruit size apparent with Baldwin when flowers were thinned there were no dramatic changes in fruit [AsA] (Fig. 3.13). Differences in the yield of ascorbic acid per bush ([AsA] × fruit yield) were due only to differences in fruit yield (Fig. 3.13).

In many crops fruit thinning has the advantage in that fruit quality (generally size) is enhanced. A measurement of the proportional distribution of fruit to three berry sizes is shown in Table 3.16. For Ben Lomond in either treatment there was little fruit in the smallest size class (5.6 to 8.2mm). In size class 8.3 to 11.2mm the proportion of fruit measured was reduced by thinning. This produced a greater proportion of fruit in the larger size class for the thinned bushes.

This increase in larger size fruit for Ben Lomond supports the level of yield compensation seen above with respect to mean total yield per bush. For Baldwin a similar pattern of

change was also apparent with an increase in the proportion of larger fruit with thinning (Table 3.22).

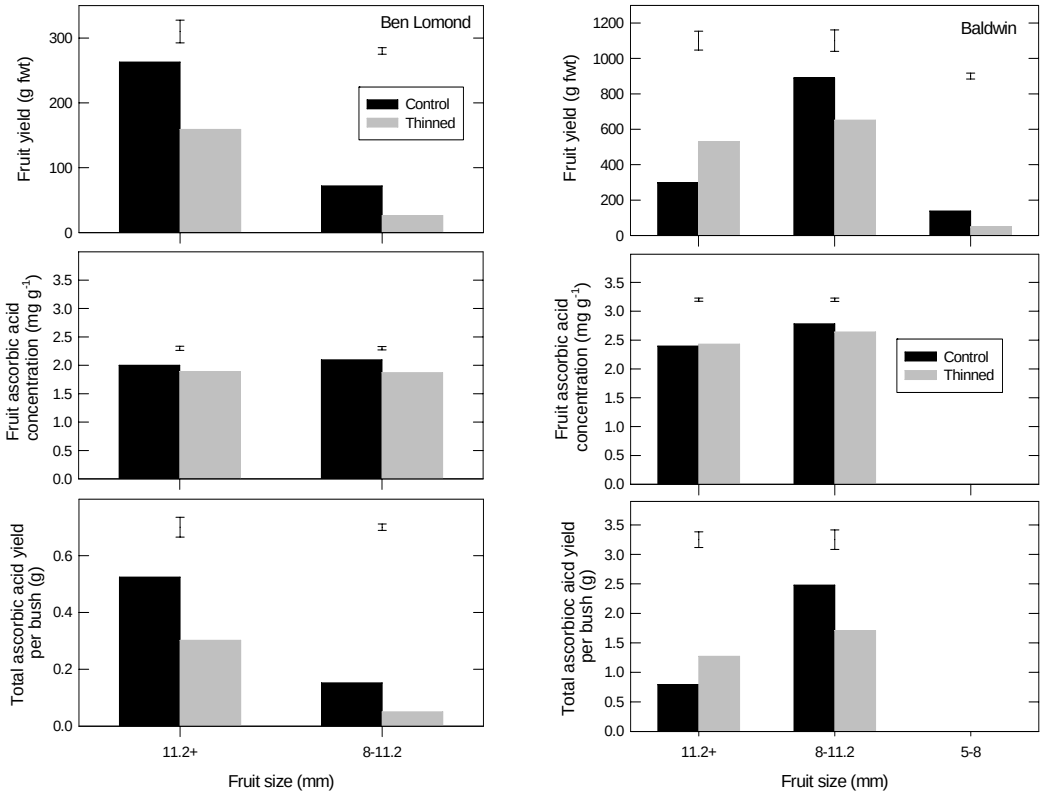


Figure 3.13 Fruit yields (g fresh weight per bush^{-1}), [AsA] (mg g^{-1}) and total amount of ascorbic acid (g per bush) per bush for the cvs Ben Lomond (left) and Baldwin (right) when thinned

Table 3.22 The influence of early fruit thinning on the proportional distribution (%) of fruit within 3 diametric size (mm) classes, for the cvs Ben Lomond and Baldwin

Cultivar	Treatment	Fruit size (mm)		
		5.6 to 8.2	8.3 to 11.2	>11.2
Ben Lomond	No thinning	<0.1	21.7	78.3
	Thinned by 50%	0.1	14.4	85.5
Baldwin	No thinning	9.9	66.3	23.8
	Thinned by 50%	4.0	52.4	43.6

However, despite there being markedly different varietal differences in proportional differences in fruit size, the impact of thinning on increasing fruit size was greater with

Baldwin. This again agrees, at least in part, with the mean total yield compensation seen above with this variety.

Effect of strig berry number (size)

The mean sizes of the individual berries on the strigs were similar, 493 mg on the large strigs and 468 mg on the small strigs. The concentration of AsA increased as berry size decreased irrespective of strig size, but for the total content of AsA per berry the reverse was true, i.e. as berry size increased AsA content increased.

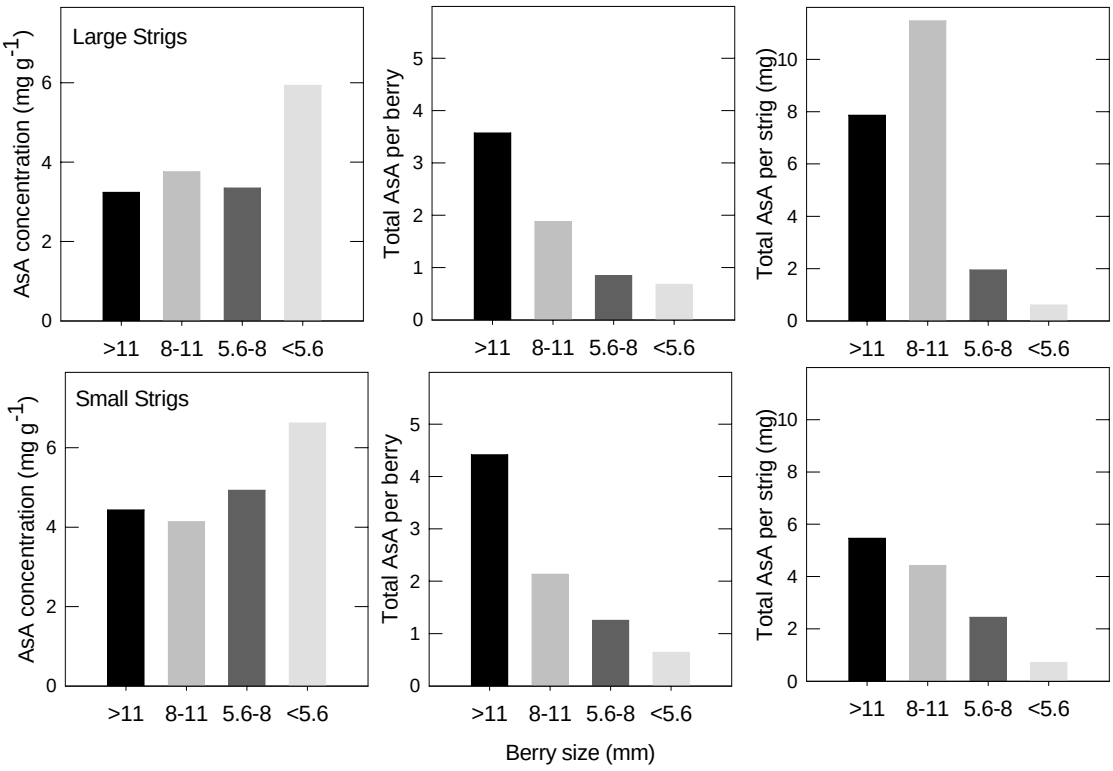


Figure 3.14 The effect of strig berry number (large = 8-14, small = 2-5) on AsA concentration (mg/g fresh weight), AsA amount per berry (mg/berry) and AsA amount per strig (mg/strig)

The mean concentration of AsA in berries on large and small strigs was 4.07 and 5.03 mg g^{-1} fresh weight respectively. When comparisons are made between berries within the same size classes, those on small strigs have greater concentrations of AsA than those on large strigs (Fig. 3.14). The mean total content of AsA per berry was 1.74 mg and 2.11 mg for the large and small strigs respectively. This effect was consistent across each berry size class on the large and small strigs (Fig. 3.14). The total AsA content in the large and small

strigs was 19 and 7 mg respectively, i.e. the greater concentration of AsA found in the berries on the smaller strigs did not compensate for the greater number of berries found on the large strigs.

Year 4 (2005)

Effects of source manipulation by strig leaf removal and branch girdling

Analyses of the fruit yields obtained on the first harvest (16 June) are shown in Fig. 3.15. These data show that between 25 May and 16 June (22 days) fruit increase in fresh weight was considerable, but leaf removal severely reduced (by 50%) mean yield or fruit number per branch. Girdling also impacted on branches with intact strig leaves, where fruit yields were 84g and 65g, for the non-girdled and girdled, respectively. While for branches with the strig leaves removed the yields were 41g and 24g for the non-girdled and girdled respectively. This suggests that around 50% of the growth over this period was likely derived from carbohydrates supplied by shoot tip leaves (extension leaves) and part of the remainder between 20 to 30% depending on treatment was from strig leaves. Presumably the remainder may have come from storage within the actual girdled branch.

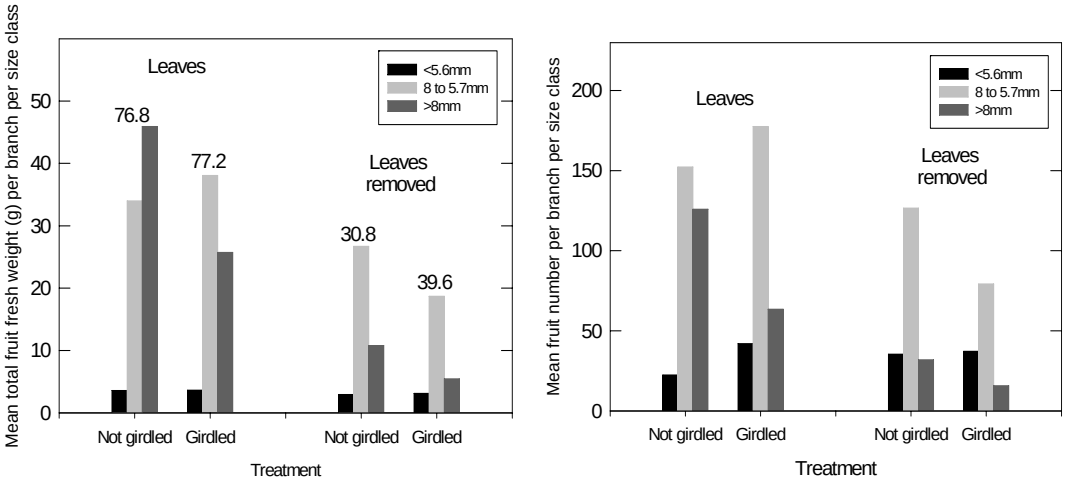


Figure 3.15 Mean total fruit fresh weights per fruit size per branch for 'Ben Tirran' bushes subject to a combination of strig leaf removal (leaves, or leaves removed) and branch basal girdling. Numbers above each treatment are the summed mean total yields per branch, irrespective of fruit size

The other samples taken were restricted to non-girdled branches, as it appears that the process of girdling isolated the branches to such an extent that they subsequently died due to lack of water.

Measurements of fruit [AsA] were less consistent compared to other experimental treatments, but there was little evidence to suggest that any of the four treatments had influenced fruit concentrations (Fig. 3.16). Differences in the yield of ascorbic acid per bush ([AsA] x fruit yield) were apparent, within a size class, but again this was due to differences in fruit yield alone.

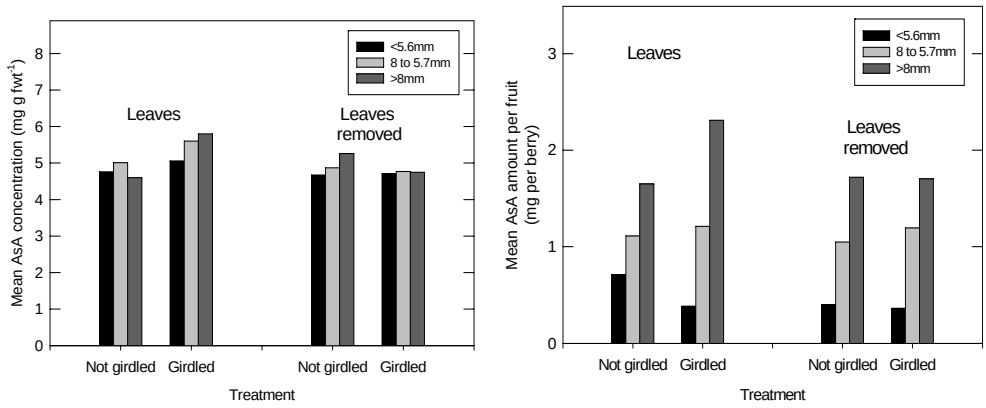


Figure 3.16 Mean fruit AsA concentration per fruit size per branch (left) and mean total amount of AsA per fruit per fruit size per branch for 'Ben Tirran' bushes subject to a combination of strig leaf removal (leaves, or leaves removed) and branch basal girdling. Numbers above each treatment are the summed mean total yields per branch, irrespective of fruit size

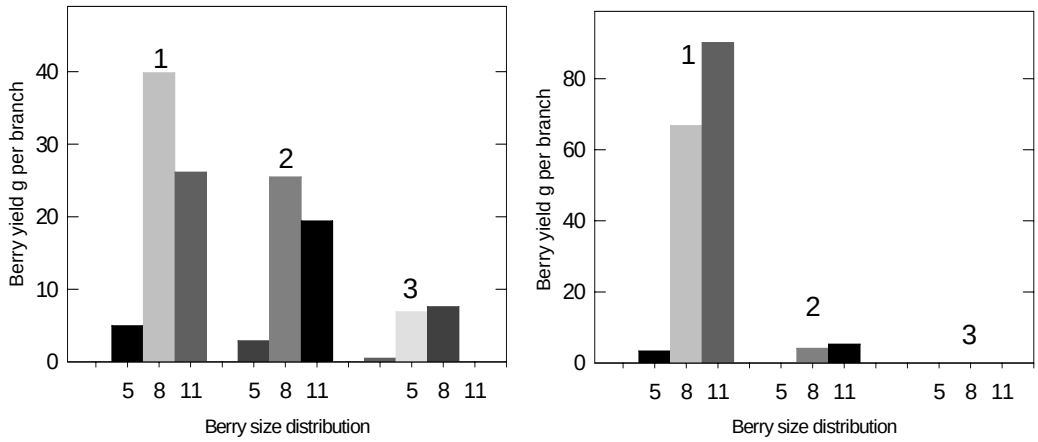


Figure 3.17 Mean fruit yields from different ages wood (years 1, 2 and 3) of Baldwin (left) and Ben Lomond' (right) from entire branches and separated into different size classes of berry (5, 8 and 11 mm diameter)

Year 5 (2006)

Determination of the impact of age of bush bearing wood on fruit [AsA]

The size and yield of fruit in relation to age of wood is shown in Fig. 3.17, for Baldwin and Ben Lomond. Age of wood has a significant impact on the distribution of fruit and fruit size. One year-old wood in the most productive followed by 2 and 3 year old respectively, irrespective of cultivar. Determination of AsA concentration showed that age of wood has no dramatic influence (Figs 3.18 and 3.19). But due to the large impact of age of wood on fruit yield there was a large effect on total AsA yield per branch for both cultivars (Figs 3.18 and 3.19).

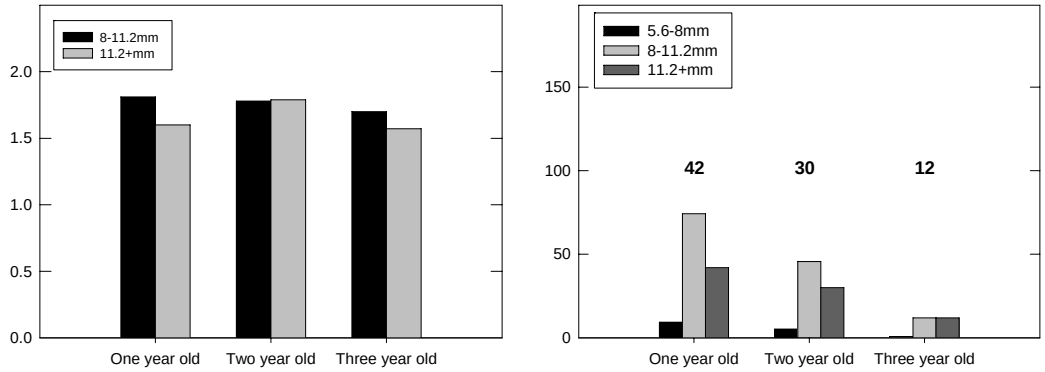


Figure 3.18 Mean fruit ASA concentration (left) and total ASA yield per branch (right) of Baldwin from different ages wood (years 1, 2 and 3) from entire branches and separated into different size classes of berry (5, 8 and 11 mm diameter)

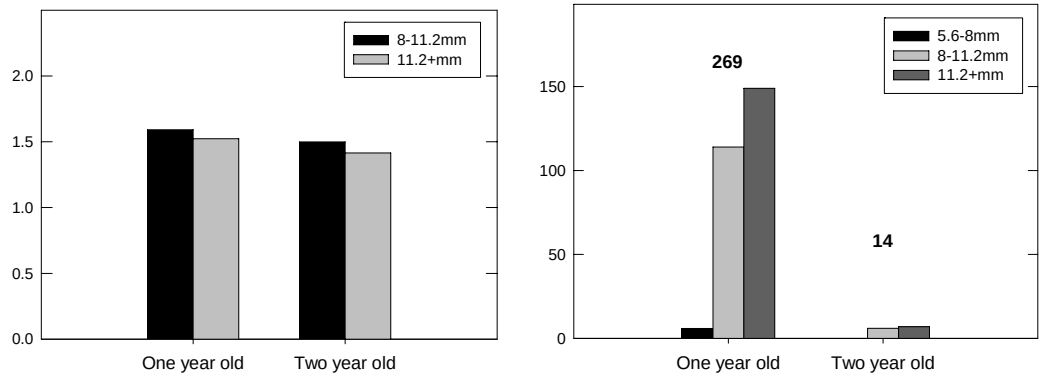


Figure 3.19 Mean fruit ASA concentration (left) and total ASA yield per branch (right) of Ben Lomond from different ages wood (years 1, 2 and 3) from entire branches and separated into different size classes of berry (5, 8 and 11 mm diameter)

Year 5 (2006)

Determination of the impact of autumn leaf defoliation on fruit [AsA] in the subsequent growing season

A comparison of AsA concentration was made on three occasions during the latter part of the development cycle of fruit collected from Baldwin and Ben Lomond bushes and is shown in Figs 3.20. The statistical analysis of this comparison is shown in Table 3.23. For both Baldwin and Ben Lomond fruit AsA concentration was low for pick 1 when the fruit were unripe, while for pick 2 the concentration was close to doubling. However, subsequently for pick 3 it returned to a concentration similar to that evident at pick 1. The decline in AsA concentration might be expected between picks 2 and 3 as the fruit expands due to water uptake. There were some statistically significant differences related to treatment, for example, smaller sized fruit in pick 1 and pick 2 for Baldwin showed greater AsA concentrations in the controls compared with the defoliated bushes (defoliated by black spot). However, the consistency of this type of interaction was not consistently apparent with all picks or cultivars. This suggests that caution should be used in interpreting these data, particularly when considering the initial limitations of the experimental design.

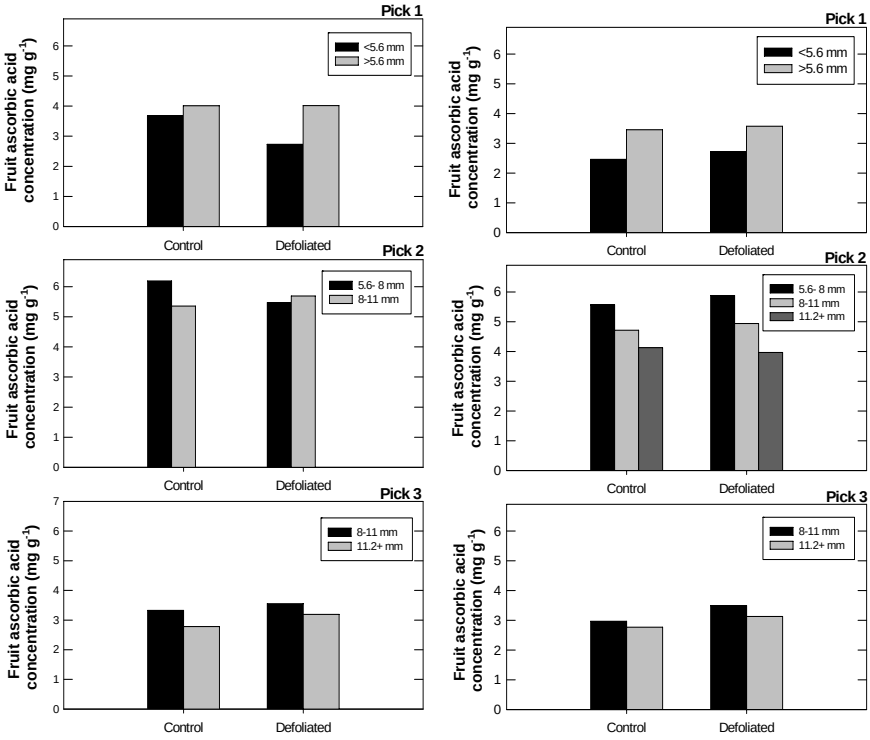


Figure 3.20 A comparison of mean fruit [AsA] (left) and total AsA yield bush^{-1} (right) of Baldwin and Ben Lomond bushes defoliated naturally, or prematurely by *Drepanopeziza ribis* (leaf spot)

Table 3.23 Statistical analysis of Baldwin and Ben Lomond AsA data contained in Figs 3.15 and 3.16. Bushes were either allowed to defoliated naturally, or prematurely by *Drepanopeziza ribis* (leaf spot)

Baldwin	*F Probability*			SED		
AsA concentration Pick 1	<5.6 0.012	>5.6 ns		<5.6 0.343	>5.6 0.277	
Baldwin	*F Probability*			SED		
AsA concentration Pick 2	5-8 0.041	8-11 ns		5-8 0.325	8-11 0.273	
Baldwin	*F Probability*			SED		
AsA concentration Pick 3	8-11 ns	11.2+ 0.026		8-11 0.126	11.2+ 0.167	
Ben Lomond	*F Probability*			SED		
AsA concentration Pick 1	<5.6 0.003	>5.6 ns		<5.6 0.075	>5.6 0.135	
Ben Lomond	*F Probability*			SED		
AsA concentration Pick 2	5.6-8 ns	8-11 ns	11+ ns	5.6-8 0.327	8-11 0.159	11+ 0.169
Ben Lomond	*F Probability*			SED		
AsA concentration Pick 3	5.6-8 -	8-11 <0.001	11+ 0.002	5.6-8 -	8-11 0.833	11+ 0.096

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Year 5-6 (2006-07)

Impact of post harvest leaf removal on subsequent fruit AsA concentration

A general pattern was apparent of an increase in fruit yield with the time bush defoliation occurred. The later leaves were removed artificially the greater the yield (Fig. 3.21). Defoliation had little influence on the proportional distribution of fruit to various sizes. There was however a small but distinctive decline in fruit [AsA] concentration associated with the delay in defoliation date. However, the major impact of the defoliation treatments was that the total yield of AsA was clearly linked to the date when leaves were removed from bushes. Delaying leaf removal/defoliation enhanced AsA yield per bush.

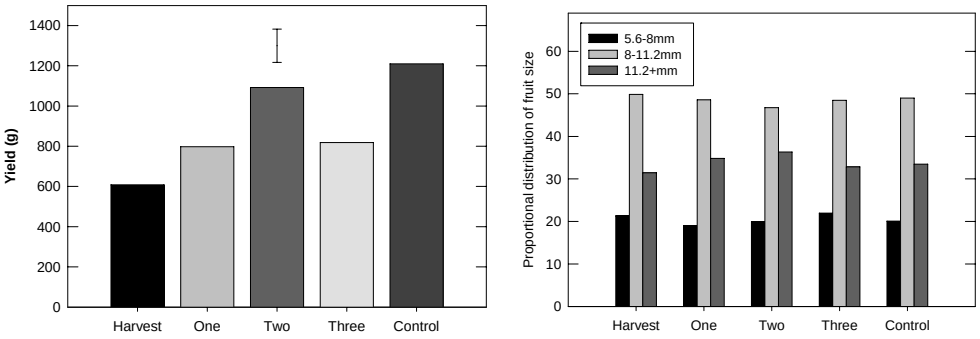


Figure 3.21 Effects of leaf removal (total bush defoliation) at different times after crop harvest on the yield and proportional distribution (%) of fruit from Baldwin bushes

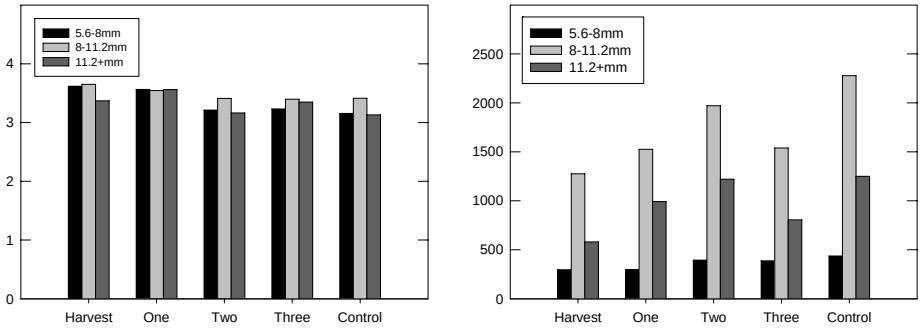


Figure 3.22 Effects of leaf removal (total bush defoliation) at different times after crop harvest on the mean fruit [ASA] (left) and total ASA yield bush^{-1} (right) of ‘fruit from Baldwin bushes

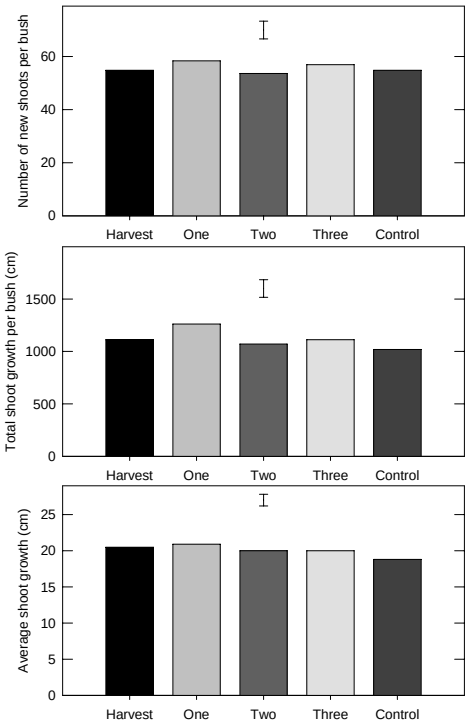


Figure 3.23 Effects of leaf removal (total bush defoliation) at different times after crop harvest on the number of new shoots, total shoot growth and the average shoot growth per shoot for bushes of Baldwin

Table 3.24 Statistical analysis of Baldwin AsA data contained in Fig. 3.18. Leaves were removed in the previous year as follows: at harvest, one month after harvest, two months after harvest, three months after harvest and a control where no leaves were artificially removed

Fruit AsA concentration	*F. Prob*			SED		
	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Treatment	ns	ns	ns	0.11	0.14	0.18

Total AsA yield per bush	*F. Prob*			SED		
	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Treatment	ns	0.02	0.06	65	308	252

Treatment	*Total AsA per bush*	*F. Prob*	SED
Harvest	2154	0.016	541
One month	2818		
Two month	3585		
Three month	2734		
Control	3965		

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Year 5-6 (2006-07)

Impact of flower removal on subsequent fruit AsA concentration

Complete flower removal the previous year from entire bushes had a highly significant positive impact on total fruit yield (Fig. 3.24). There was, however, little influence on the distribution of berries to the different size categories (Fig. 3.24).

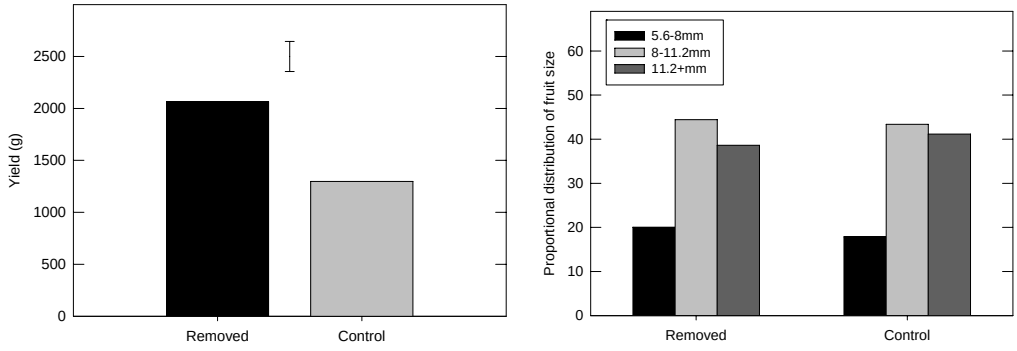


Figure 3.24 Effects of complete flower removal the previous year from the entire bushes of Baldwin on the yield of fruit and the distribution of fruit to different size categories

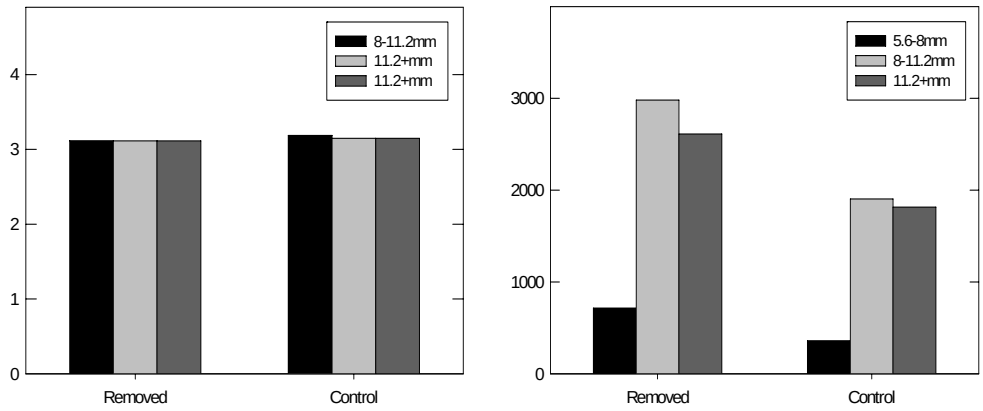


Figure 3.25 Effects of complete flower removal the previous year from the entire bushes of Baldwin on [AsA] (left) and the total yield of AsA bush^{-1} (right)

Measurements of [AsA] of berries derived from bushes that had been previous deflowered showed no treatment or size category differences (Fig. 3.25). However, when the total yield of AsA bush^{-1} was determined by multiplying the berry concentration of AsA by the bush yield there was a marked positive impact on total AsA yield (Fig. 3.25). The enhanced yield obtained from the bushes that had been previously deflowered was statistically significant. This was accompanied by an increase in shoot growth (Fig. 3.26).

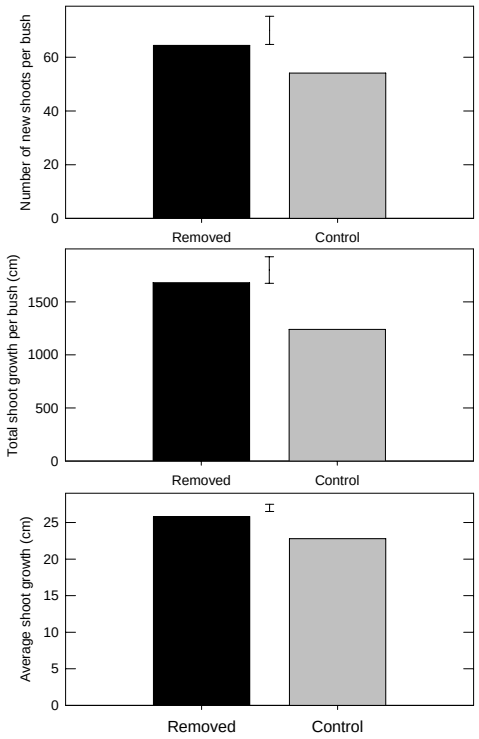


Figure 3.26 Number of new shoots, total shoot growth and the average shoot growth per shoot for bushes of Ben Lomond and Baldwin when grown for one year after complete flower removal the previous spring

Table 3.25 Statistical analysis of Baldwin AsA data contained in Fig. 3.21. Flowers were removed in the previous year as follows: either removed or not removed

Fruit AsA concentration		*F. Prob*			SED	
	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Treatment	ns	ns	ns	0.11	0.08	0.08

Total AsA yield per bush		*F. Prob*			SED	
	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Treatment	0.027	0.035	ns	135	435	426

Treatment	*Total AsA per bush*	*F. Prob*	SED
Flowers removed	6306	0.043	949
Control	4077		

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Enhancing the concentration AsA through spray applications of AsA biosynthetic precursors

Year 3 (2004)

The results obtain from this experiment are shown in Table 3.26. The effects of galactonic acid cannot be easily interpreted. The results obtained with the double spray 24 h apart show no significant treatment responses in relation to bush position or berry size. If a double application of galactonic acid failed to alter AsA berry concentration, then it is difficult, physiologically, to understand why a single application did enhance AsA for the smaller size class of berry, as was the case here (Table 3.26). It remains unclear if the precursor does influence AsA concentration, but clearly we have no evidence that it was taken up by the plant, so this might explain the lack of a response or its variability. Applications of the precursor(s) at different fruit developmental stages might prove to be more effective and may be worth trying.

Table 3.26 The effects of field L-galactono-1,4-lactone acid sprays (25mM) on the AsA concentration of berries. Sprays were applied at 24 h intervals and fruit were collected for AsA analysis after a further 24 h delay

Treatment	Berry size (mm)	Mean AsA concentration mg g^{-1}	
		Water plus Tween	L-galactono-1,4-lactone plus Tween
1 spray application			
Top of branch	<11	3.42	3.38
	8-11	3.34	4.83
Lower branch	<11	3.53	3.40
	8-11	3.83	3.90
2 spray applications			
Top of branch	<11	3.11	3.33
	8-11	3.21	3.05
Lower branch	<11	2.93	2.88
	8-11	3.41	3.34

Non-destructive method to determine* in situ *[AsA] in fruit by micro-dialysis AsA concentration in detached berries

Initial results suggest that the probes, despite the suggestions on the manufactures, can have a multi-use approach (used on more than one fruit). The concentrations of AsA found in fruits using the probes were similar to those reported from conventional methods. Also, effective flow rates and perfusion volumes were obtained from these initial replicated experiments. In all these experiments fruit of similar developmental age and position of the 'strig' were selected, i.e. mature, large fruit at the top of the strig.

The AsA concentration measured in the dialysate from detached berries of the cv. Ben Connan is shown in Fig. 3.27. Dialysate from 6 individual berries examined using micro-dialysis probes shows that initially the concentration of berry AsA was high but rapidly fell within the first hour of measurement. This is a likely reflection of the high concentration achieved as the probes are mounted in the berry. The placement of the probe causes cell damage and disruption and the release of cellular and vacuolar contents. This disruption appears to cause a release of AsA, probably stored in the vacuole. Once this initial AsA contamination has been flushed away (>60 min), AsA concentration remains constant for several hours. The initial success of this technique warrants further development with the potential of the approach to be used as a tool to examine the site of AsA synthesis and possible transport routes.

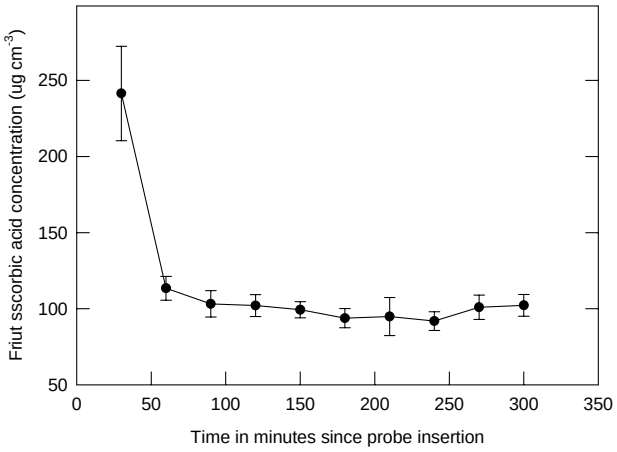


Figure 3.27 The concentration of AsA measured in dialysate from detached berries of the cv. Ben Connan

Analysis of AsA in berries pre-treated with L-galactono-1,4-lactone

Micro-dialysis probes were used to determine if pre-soaking, for 24 h, detached berries of the cv. Ben Connan in a 25mM solution of L-galactono-1,4-lactone would enhance AsA concentration over a 5 hour period. The results presented in Fig. 3.28 show that the extractable concentration of AsA declined with time, but there was no evidence that pre-soaking of berries with L-galactono-1,4-lactone in anyway changed the shape of the curves presented. This supports the evidence found with the field experiments where this precursor to AsA synthesis also showed no obvious influence on AsA berry concentration.

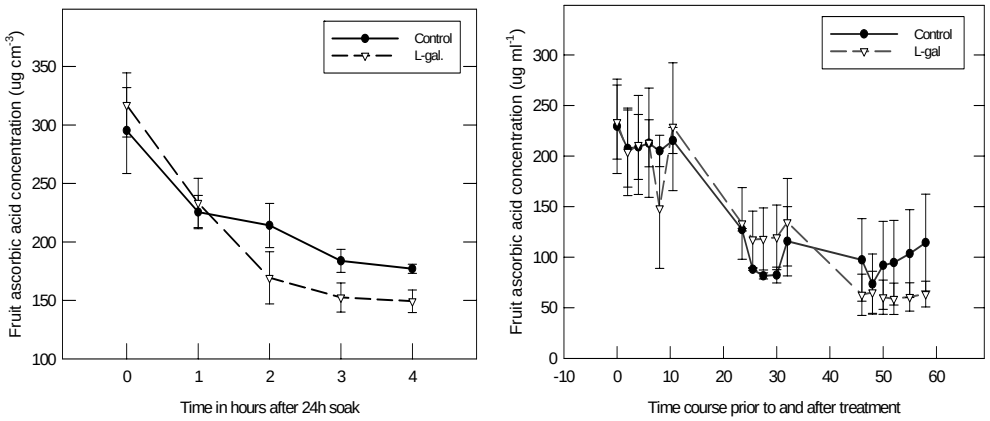


Figure 3.28 The concentration of AsA measured in dialysate from detached (left and attached (right) berries of the cv. Ben Connan after either soaking for 24h or spraying with L-galactono-1,4-lactone (galactonic acid)

A similar experiment has been carried out while berries were still attached to the bush, and the precursor, L-galactono-1,4-lactone, was sprayed directly on the leaves as in the field

experiment. The results obtained, with micro-dialysis, suggest that over a 15 h period there was no precursor influence on berry AsA concentration.

Manipulation of nitrogen supply

Year 2 (2003)

Application of different nitrogen concentrations to potted bushes

Weekly analyses of leaf nitrogen content (as estimated by the chlorophyll content meter), and the final chlorophyll content reading and leaf mineral analyses at the time of fruit harvest, are shown below. The data shows that the 5 nitrogen treatments were grouped within three categories, viz. low nitrogen (treatment 1), medium nitrogen (treatment 2), and high nitrogen (treatments 3-5). This effect is evident irrespective of cultivar, and can be seen from the seasonal presentation of the chlorophyll content data in Fig. 3.29.

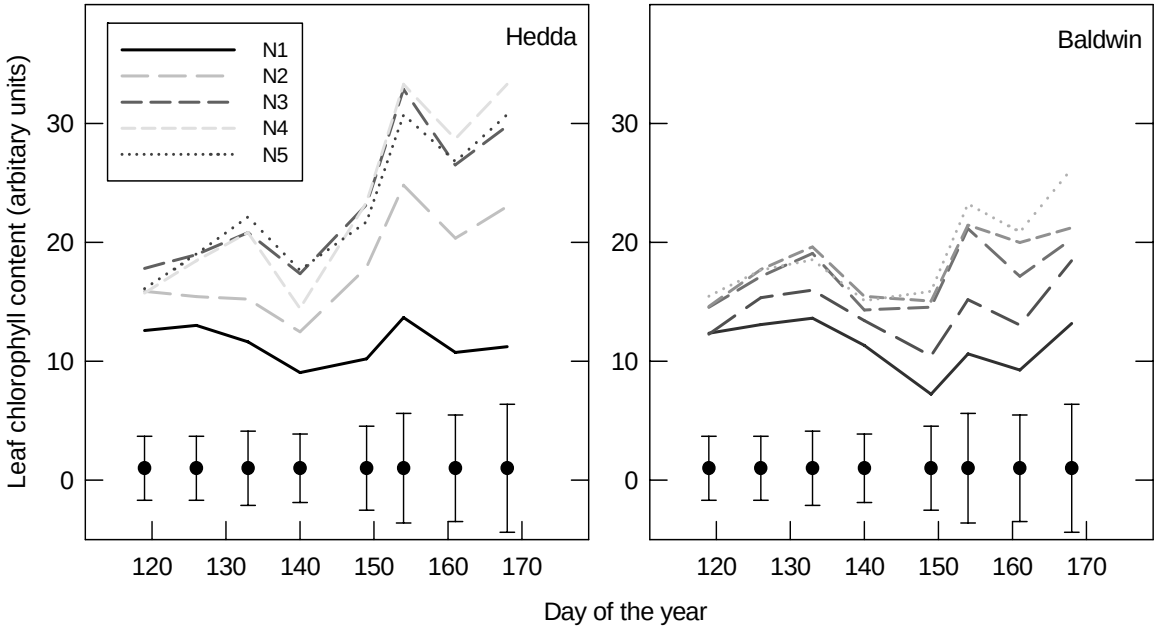


Figure 3.29 Non-destructive measurements of **c**hlorophyll content (arbitrary units) of leaves from Hedda and Baldwin used as a proxy to estimate leaf nitrogen content during the growing season and final leaf nitrogen analyses at the time of fruit harvest

A comparison of final leaf nitrogen content with recommended concentrations indicates that the low, medium and high nitrogen treatments applied in this experiment successfully

achieved a range of nitrogen fertilisation spanning nitrogen-deficiency to nitrogen-oversupply (Table 3.27). A comparison of leaf chlorophyll content against directly extractable leaf chlorophyll concentration is shown in Fig. 3.30.

Table 3.27 Experimental (E) and recommended optimal (R) values of leaf nitrogen concentration as measured experimentally and obtained from published results*, respectively

Nitrogen treatment	Leaf nitrogen concentration (% nitrogen)		(E/R) × 100
	Mean experimental value (E)	Optimal range* (R)	
Low nitrogen (N1)	1.76	2.9 - 3.0	60 %
Medium nitrogen (N2)	3.10	2.9 - 3.0	105 %
High nitrogen (N3-5)	3.83	2.9 - 3.0	130 %

* http://www.hortnet.co.nz/publications/guides/fertmanual/black.htm

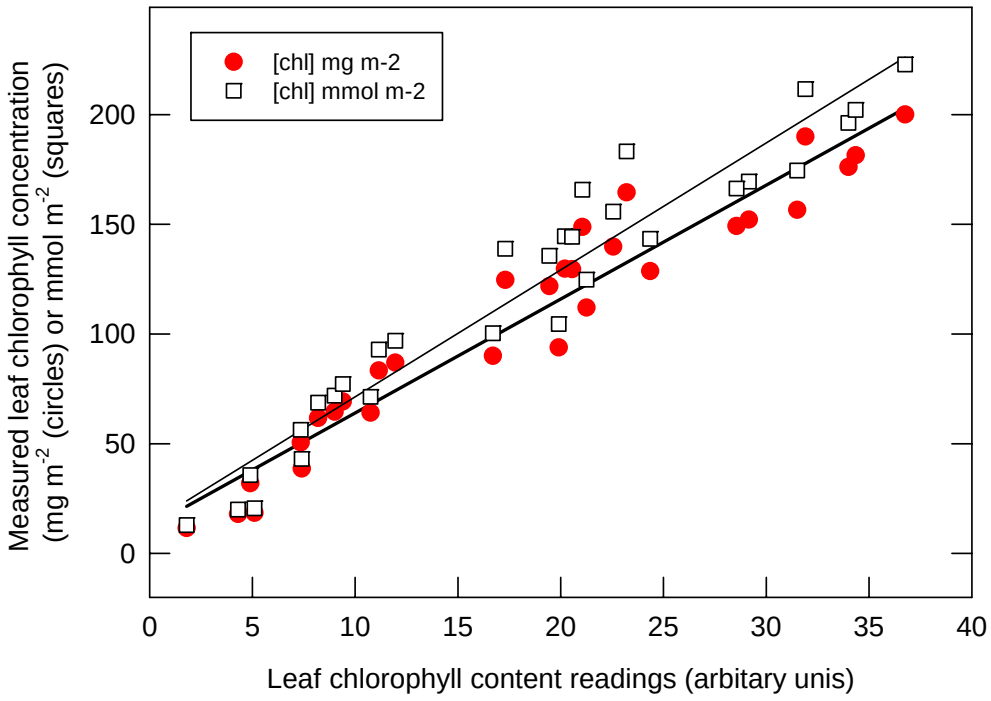


Figure 3.30 The relationship between non-destructive measurements of **c**hlorophyll content (arbitrary units) of leaves from Baldwin and direct destructive chemical measurement of leaf chlorophyll concentration expressed as mg m^{-2} or mmol m^{-2}

Analyses of other leaf minerals showed that these were generally within, or slightly above, the optimal range (but see comments on phosphorus) (Fig. 3.31).

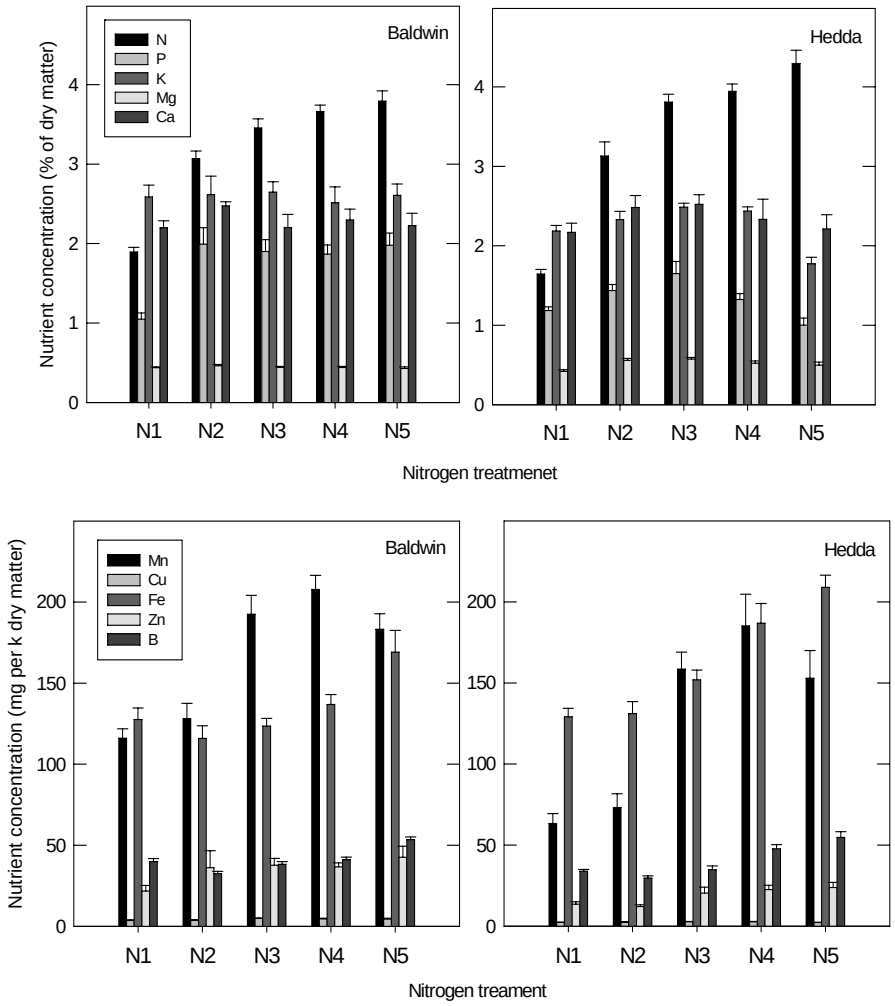


Figure 3.31 Macro- and micro-nutrient analyses of leaves of Baldwin (left) and Hedda (right) plants grown in pots supplied with different nitrogen regimes

The high phosphorus values were most likely artefactual (possibly due to pesticide sprays applied to the bushes to control mildew and leaf midge). The fertigation regime applied as described in the experimental section was based on a standard solution culture formulation. This was not adjusted to provide high phosphorus levels, and the bushes showed no signs of phosphorus toxicity throughout the experiment. Furthermore, there was no deficiency in calcium, which might have been expected, via precipitation of calcium phosphate, if excess phosphorus had been applied to the bushes. Soil pH was monitored throughout the experiment to check ammonium assimilation did not lead to adverse soil acidification.

Blackcurrants perform best with a soil pH above 5.8, as indicated in published results (see http://www.hortnet.co.nz/publications/guides/fertmanual/black.htm). Treatments N3-N5 showed soil acidification below pH 5.8 towards the end of May 2003, and so the soil was treated with a lime suspension.

Table 3.28 Experimental (E), averaged over treatments 1-5, and recommended optimal (R) values of leaf nitrogen concentration as measured experimentally or obtained from published results*

Mineral	Concentration units	Mean experimental value (E)	Normal optimal range (R)
Phosphorus	%	1.55	0.26 - 0.30
Potassium	%	2.42	1.5 - 2.0
Calcium	%	2.32	2.0
Magnesium	%	0.48	0.15 - 0.60
Manganese	ppm	146	30 -100
Iron	ppm	148	50 -100
Zinc	ppm	27.3	20 - 40
Copper	ppm	3.38	5 - 10
Boron	ppm	40.4	20 - 40

* http://www.hortnet.co.nz/publications/guides/fertmanual/black.htm

Table 3.29 The effects of nitrogen application concentration on final crop harvest and the proportion of berries of different size categories from Hedda and Baldwin bushes

Treatment (ppm N)	Total crop yield (g)			% fruit <8 mm			% fruit 8-11.2 mm			% fruit >11.2 mm		
	H	B	M	H	B	M	H	B	M	H	B	M
Nitrogen 1 (19 ppm)	126	123	125	3	4	3	47	53	50	50	44	47
Nitrogen 2 (131 ppm)	202	148	175	0	0	0	20	27	23	80	73	76
Nitrogen 3 (243 ppm)	228	172	200	0	1	0	6	22	14	94	77	86
Nitrogen 4 (467 ppm)	191	159	175	0	1	1	5	31	18	95	68	81
Nitrogen 5 (915 ppm)	216	137	177	0	0	0	8	24	16	92	76	84
P value Cultivar:	*0.20*			*0.02*			*0.003*			*0.003*		
Nitrogen conc:	*0.31*			*<0.001*			*<0.001*			*<0.001*		
Cultivar x N-conc:	*0.85*			*0.47*			*0.13*			*0.13*		

Note: H, Hedda; B, Baldwin; M, mean (average value for both cultivars). The percentage values refer to the total fruit crop (ripe and unripe) within size categories as defined by berry diameter in mm.

Cultivar Hedda produced a larger crop yield than cv. Baldwin for each treatment level (Table 3.29), although this was not statistically significant (P = 0.20). There was no

statistically significant overall N-treatment effect on crop yield ($P = 0.31$), although it can be seen that the total crop yield was smaller for treatment N1 than for the yields of treatments N2-N5, for both Hedda and Baldwin. There were significant effects of cultivar ($P \leq 0.02$ for angular transformed percentages) and N-treatment ($P < 0.001$ for angular transformed percentages) on the proportion of fruits in the various size categories. Hedda produced a greater proportion of large fruit (>11.2 mm diameter) than Baldwin. As nitrogen fertigation increased from N1-N5, the proportion of larger fruit (>11.2 mm diameter) tended to increase, whereas the proportions of small (<8 mm) and medium (8-11.2 mm) fruit tended to decrease.

Table 3.30 The effects of nitrogen application concentration on the dry matter partitioned to total bush leaf area and the percentage allocated to new shoot growth (current season shoot) or leaves to Hedda and Baldwin bushes

Treatment (ppm N)	Total leaf area (m^2)			% new shoots			% leaves		
	H	B	M	H	B	M	H	B	M
Nitrogen 1 (19ppm)	0.5	0.6	0.5	9	5	7	15	18	16
Nitrogen 2 (131ppm)	0.9	0.7	0.8	13	14	14	24	28	26
Nitrogen 3 (243ppm)	1.1	1.1	1.1	14	15	15	27	31	29
Nitrogen 4 (467ppm)	1.2	0.9	1.1	15	15	15	31	30	30
Nitrogen 5 (915ppm)	0.9	0.9	0.9	9	14	11	29	34	31
P value									
Cultivar:			*0.75*			*0.69*			*0.03*
Nitrogen concentration:			*<0.001*			*<0.001*			*<0.001*
Cultivar x N concentration:			*0.47*			*0.01*			*0.15*

Note: H, Hedda; B, Baldwin; M, mean (average value for both cultivars)

There was no significant cultivar × nitrogen treatment interaction, which confirms that both cultivars responded in a similar fashion to nitrogen treatment. Nitrogen treatment did not significantly affect the proportion of ripe fruit at harvest (data not shown).

Nitrogen-treatment also had a significant effect on bush total leaf area and percentage of dry matter allocation within leaves and new shoot growth (Table 3.30), with increasing nitrogen tending to increase these values, with the most marked comparisons being between N1 and N2-N5.

The level of nitrogen application had a significant effect on [AsA]. Results for ripe berries of the larger size category (diameter >11.2 mm) are shown in Fig. 3.32.

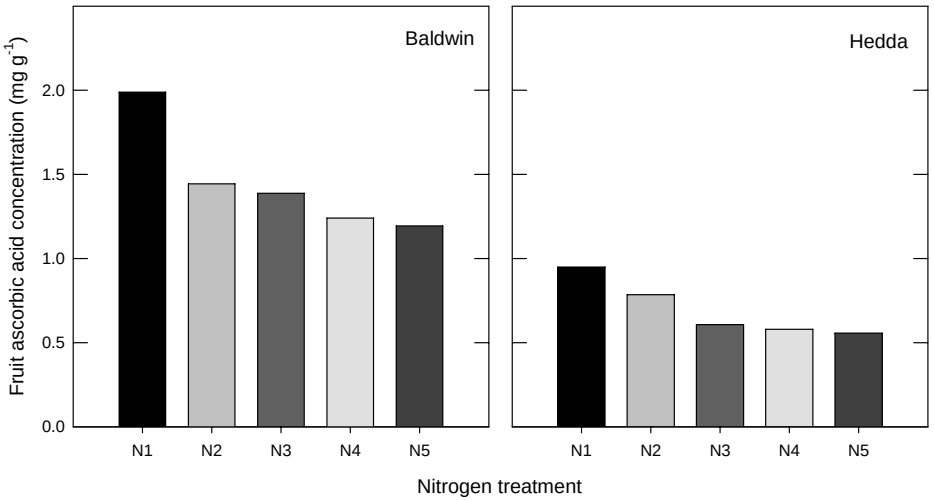


Figure 3.32 Effects of different concentrations of applied nitrogen on [AsA] on berries >11.2 mm diameter for the cvs Hedda and Baldwin

There was a significant cultivar effect (P <0.001), with berries from Hedda displaying approximately half the [AsA] found in Baldwin. Also, there was a significant treatment effect (P <0.001), with [AsA] declining as nitrogen application increased from N1-N5. In terms of the significance of individual differences, the results for Hedda could be grouped as: N1, N2, N3-N5, and the results for Baldwin as N1, N2-N3, N4-N5.

The large berries (>11.2 mm) formed the largest proportion of the crop (Table 3.4), and thus the statistical analyses of [AsA] were performed on the ripe berries in this size range only. There were not complete data sets for the smaller sized berries, but the [AsA] declined with increasing nitrogen application for these berries also (data not shown).

Year 3 (2004)

Application of different nitrogen concentrations to field-grown bushes

Chlorophyll content did not differ significantly between the treatments, throughout the period that measurements were conducted (Fig. 3.33). This implies that the N concentration in the leaves was unaffected by nitrogen application rates ≥76 kg N ha^{-1}. The 'standard' application rate (76 kg N ha^{-1}) was sufficient also to sustain shoot growth as more N had no impact on total shoot length, number of new shoots and average shoot

length (Table 3.31). This lack of growth response was also reflected in harvest yield (Table 3.32), as increasing N application rates had no effect on total number of berries and harvest yield of berries per bush or the size distribution of the berries.

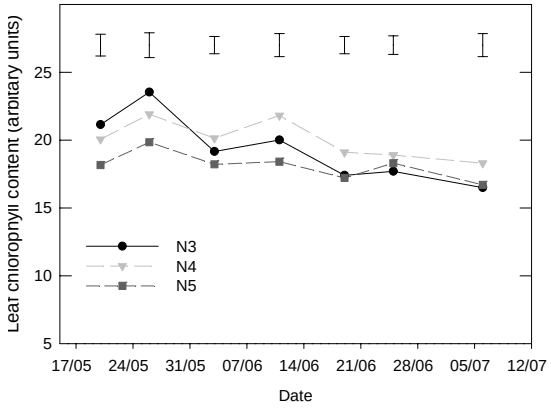


Figure 3.33 The effect of nitrogen application on the chlorophyll content (arbitrary units) of extension Baldwin leaves during 2004

The greatest [AsA] occurred within the mid sized (i.e. 8-11.2 mm diameter) berries. Increasing N application decreased [AsA] in the largest (>11.2 mm) and smallest (5-8 mm) berries, but the effects only approached statistical significance for the former (Table 3.33). Raising the application of N >76 kg N ha^{-1} had no effect on the total bush yield of AsA (Table 3.34).

Table 3.31 The effects of nitrogen application to Baldwin bushes on vegetative shoot growth

Treatment	Number of shoots	Total shoot length (cm)	Mean shoot length (cm)
N3 (76 kg N ha^{-1})	24	820	34
N4 (152 kg N ha^{-1})	25	835	34
N5 (304 kg N ha^{-1})	27	874	32
SED (6 d.f.)	*3.2*	*91.5*	*2.6*
P	*0.579*	*0.837*	*0.574*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.32 The effect of nitrogen application on total crop per Baldwin bush and the proportion of berries in different size categories

Treatment	Fruit size class (diameter mm)						Total
	>11.2		8-11		5-8		
	Yield (g)	%	Yield (g)	%	Yield (g)	%	Yield (g)
N3 (76 kg N ha^{-1})	354	39	517	58	21	3	892
N4 (152 kg N ha^{-1})	425	42	541	56	19	2	984
N5 (304 kg N ha^{-1})	326	39	505	59	19	2	849
SED (6 d.f.)	*69.0*		*90.7*		*4.99*		*156.2*
P	*0.395*	*0.455*	*0.922*	*0.538*	*0.930*	*0.954*	*0.693*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.33 The effects of nitrogen application on Baldwin berry AsA concentration (mg g^{-1} fresh weight)

Treatment	Fruit size class (diameter, mm)		
	>11.2	8-11.2	5-8
N3 (76 kg N ha^{-1})	2.38	2.94	2.46
N4 (152 kg N ha^{-1})	2.30	2.80	2.25
N5 (304 kg N ha^{-1})	2.29	2.98	2.28
SED (6 d.f.)	*0.039*	*0.147*	*0.196*
P	*0.10*	*0.49*	*0.55*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 3.34 The effects of nitrogen application on total Baldwin bush AsA content (mg $bush^{-1}$)

Treatment	Fruit size class(diameter, mm)			Total
	>11.2mm	8-11.2mm	5-8mm	
N3 (76 kg N ha^{-1})	836	1511	49	2400
N4 (152 kg N ha^{-1})	968	1513	541	2520
N5 (304 kg N ha^{-1})	739	1577	44	2360
SED (6 d.f.)	*155.0*	*349.4*	*9.3*	*477.0*
P	*0.32*	*0.98*	*0.69*	*0.94*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Despite Baldwin fruit yields being greatest for the treatment which received no additional nitrogen (N1) compared to the highest (N5), the impacts of nitrogen application during the growing season were not particularly obvious (Fig. 3.34). For Ben Lomond fruit yields appear to be slightly greater for the N3 treatment. This greater yield for N3 Ben Lomond bushes was statistically significant, with leaf chlorophyll content measurements, however, being in the middle of the range measured for all the treatments (Fig. 3.35).

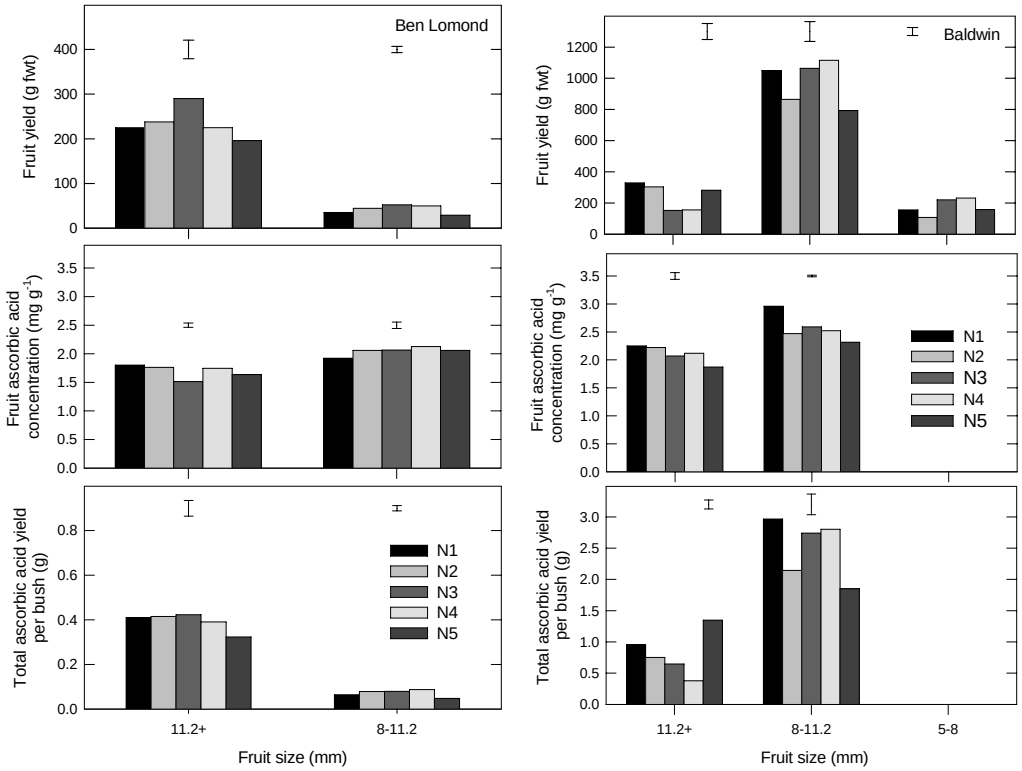


Figure 3.34 Fruit yields (g fresh weight bush^{-1}), [AsA] (mg g^{-1}) and total amount of ascorbic acid (g bush^{-1}) for the cvs Ben Lomond (left) and Baldwin (right) when grown with different nitrogen (calcium ammonium nitrate) fertiliser supply rates

Note: N1, no application; N2, 38kg nitrogen ha^{-1}; N3, 76kg nitrogen ha^{-1}; N4, 152kg nitrogen ha^{-1} and N5, 304 kg nitrogen ha^{-1}

This suggests that there is no simple relationship between nitrogen application and leaf chlorophyll content as measured here (Figs 3.34 and 3.35). This may in part be due to the

fact that the methodology used made repeated measurements of the same leaf and if leaves develop and senesce at different rates in response to nitrogen (this is documented with other species) then this can influence the results. Equally there was no obvious time dependent relationship between leaf chlorophyll content and time of application; again this may be due to differences in the pattern of nitrogen allocation and its redistribution to actively growing shoot tips and leaves (Fig. 3.35).

Fruit [AsA] were generally very consistent with cultivars and berry size irrespective of cultivar (Fig. 3.34). There were however small statistically significant differences between treatments, which suggests a gradually decline in [AsA] with increasing nitrogen fertilisation. When calculating the yield of ascorbic acid per bush ([AsA] × fruit yield) there was a slight trend for ascorbic acid yield to decline with increased nitrogen application (Fig. 3.34).

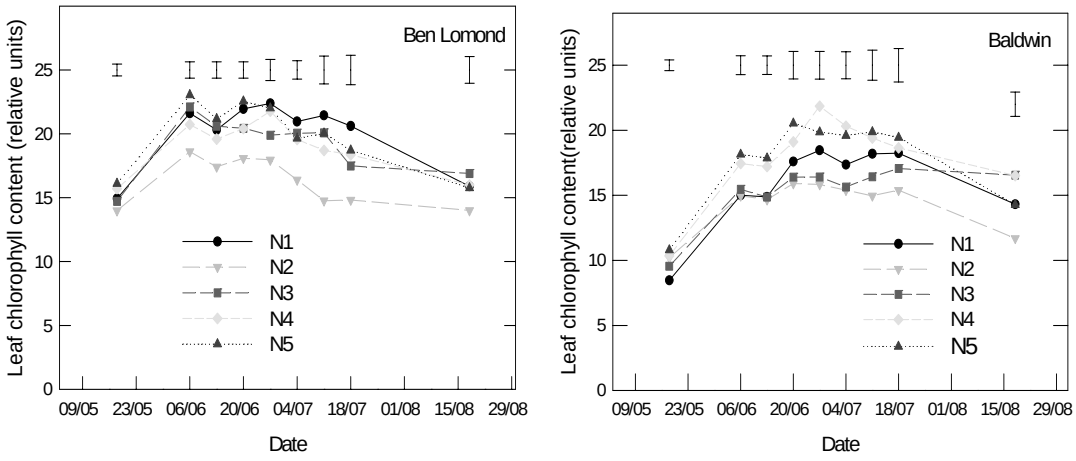


Figure 3.35 Leaf chlorophyll content (relative units) measured repeatedly on the same leaf throughout the growing season for the cvs Ben Lomond (left) and Baldwin (right) bushes fertiliser with increasing amount of ammonium nitrate, i.e. increasing from N1 to N5

Objective 4 - Identify agronomic practices for optimisation for AsA crop yield

Rationale

The primary aim of objective 4 was to develop successful methodologies identified in objective 3 that could be adapted as practical and cost-effective agronomic techniques that could be used by growers to enhance blackcurrant fruit AsA concentration.

Highlights

- Phosphorous nutrition had little on fruit yields, size distribution or AsA concentrations

Manipulation of bush phosphorus concentration to enhance fruit [AsA]

Year 3 (2004)

Seniphos application during 2004 did not influence AsA concentration of berries, but slightly increased Brix at both sites (Table 4.1).

Table 4.1 The effect of bush phosphorus enhancements sprays, through the application of 'Seniphos', on berry [AsA] and Brix

Site	**[AsA] concentration (mg g^{-1} fresh weight)**		**Brix (% soluble solids)**	
	Horsmonden	**Matfield**	**Horsmonden**	**Matfield**
No 'Seniphos'	3.37	3.43	16.3	17.4
'Seniphos'	3.33	3.30	15.7	16.5
	P	*SED (36 d.f.)*	*P*	*SED (36 d.f.)*
Site	*0.807*	*0.065*	*0.006*	*0.33*
'Seniphos'	*0.182*	*0.065*	*0.042*	*0.33*
Site × 'Seniphos' (interaction)	*0.493*	*0.092*	*0.672*	*0.46*

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

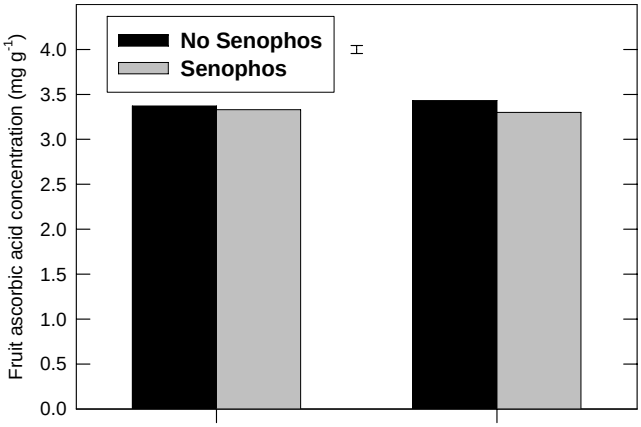


Figure 4.1 The ascorbic acid concentration in fruit of the cultivar Ben Hope grown on two sites Horsmonden and Matfield belonging to Overy Farms, after seasonal treatment with *Seniphos*™ (Phosyn plc

Application of Seniphos™ at rates used on other crops, to bushes of Ben Hope, at two grower farms has no influence on fruit [AsA] measured at harvest (Figure 4.1).

Manipulation of bush phosphorus concentration to enhance fruit [AsA]

Overy Farms grower trial – spray manipulation using 'Seniphos'

Year 4 (2005)

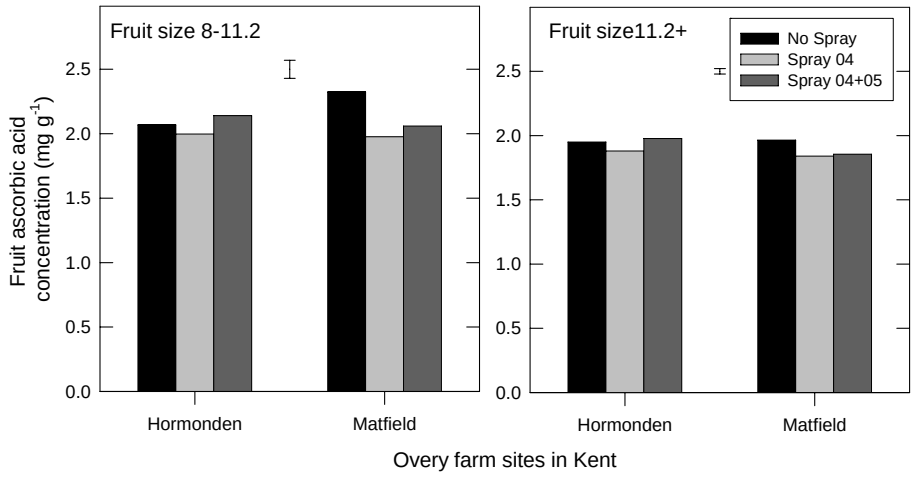


Figure 4.2 The [AsA] in fruit of the cv. Ben Hope grown on two sites Horsmonden and Matfield belonging to Overy Farms, after seasonal treatment with *Seniphos*™ (Phosyn plc)

Measurements of fruit ascorbic acid show no evidence that repeated foliar sprays, during the growing season, have any impact on [AsA], at either site (Fig. 4.2). Treatments in 2005 were slightly different than in 2004; where in the first year of application there were also no effects of Seniphos™ application on [AsA]. In 2005 the second year of application part of the area of crop treated in 2004 was treated again, but this repeated application did not show any change in [AsA].

Manipulation of bush potassium phosphite concentration to enhance fruit [AsA]

Year 4 (2005)

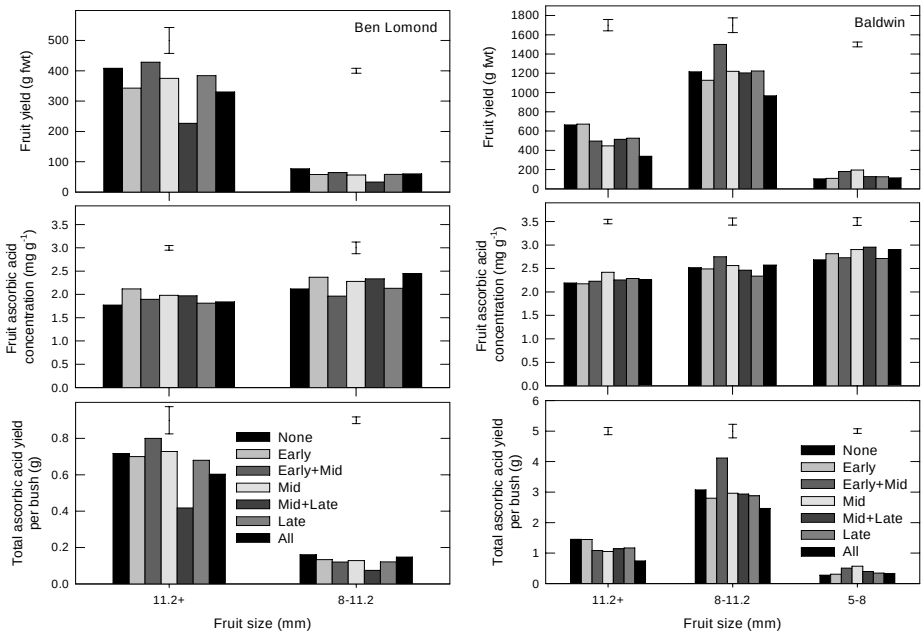


Figure 4.3 Mean total fruit fresh weights for Ben Lomond (left) and Baldwin (right) bushes when subject to application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard commercial recommendation rate of 10 litres hectare^{-1}

None, No application of Farm-Fos; E, Early application of Farm-Fos; EM, Early and mid application of Farm-Fos; Mid, Mid application of Farm-Fos; ML, Mid and late application of Farm-Fos, All, early, mid and late application Farm-Fos

The results from repeated applications of the fertilizer Farm-Fos-44 are shown in Fig. 4.3. Mean fruit yields per bush vary with cultivar used and are much lower for the younger Ben Lomond bushes, but there does not appear to be any treatment effects, for either cultivar

(Fig. 4.3). There was a significant consistent negative influence on fruit yield of Baldwin. Measurements of [AsA], as with other experiments, were consistent within a cultivar and differed little with fruit size (Fig. 4.3). Determination of total yields of ascorbic acid per bush (fruit [AsA] × fruit yield) were due only to treatments differences in fruit yield. As with the determination of mean total yield per bush (Fig. 4.4) there were no obvious Farm-Fos treatment effects on the proportional fruit size distribution (Fig. 4.5)

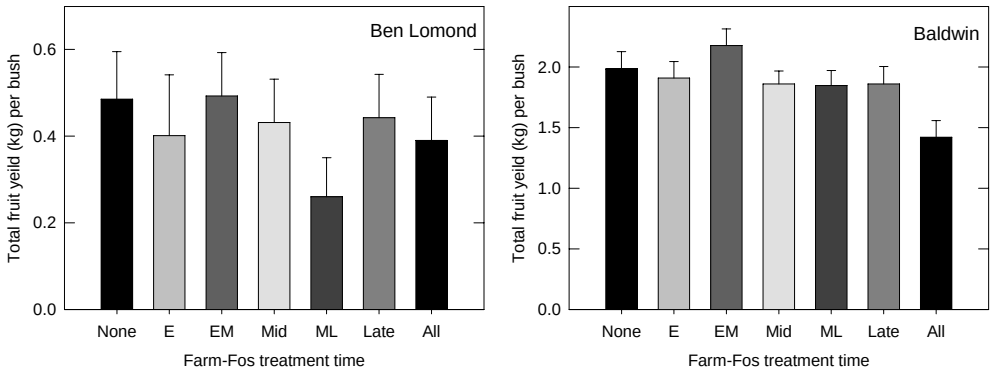


Figure 4.4 Mean fruit yields per bush of Ben Lomond and Baldwin bushes when subject to application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard commercial recommendation rate of 10 litres hectare^{-1}

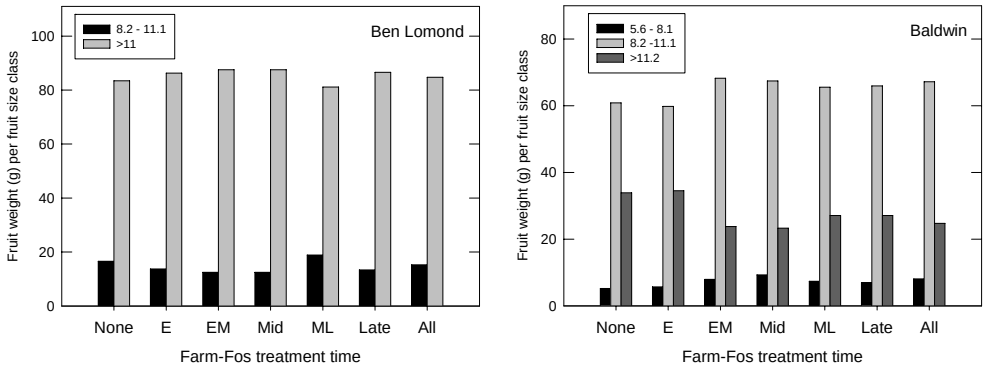


Figure 4.5 Mean proportional distribution of fruit fresh weights to various fruit sizes for Ben Lomond (left) and Baldwin (right) bushes when subject to application of Farm-Fos-44 [32% P_2O_5 and 29% K_2O] using standard commercial recommendation rate of 10 litres hectare^{-1}

None, No application of Farm-Fos; E, Early application of Farm-Fos; EM, Early and mid application of Farm-Fos; Mid, Mid application of Farm-Fos; ML, Mid and late application of Farm-Fos, All, early, mid and late application Farm-Fos

Determination of the potential for bush concentration of potassium phosphate to enhance fruit [AsA]

Fruit yields of both Baldwin and Ben Lomond showed no obvious or statistically supportable differences that could be attributed to the application of Farm-Fos in 2006 (Figs 4.6 and 4.9). Equally, there were no differences, for both cultivars, in the proportional allocation of fruit to different size categories, which could be linked the different treatments (Figs 4.7 and 4.10). Again, despite small cultivar differences in mean fruit [AsA] and total AsA per bush there were few significant differences which could be linked to treatment (Figs 4.7 and 4.10). The details of the AsA statistical analysis are shown in Table 4.2. There were no detectable differences in mean fruit soluble solids or citric acid concentration (Figs 4.8 and 4.11).

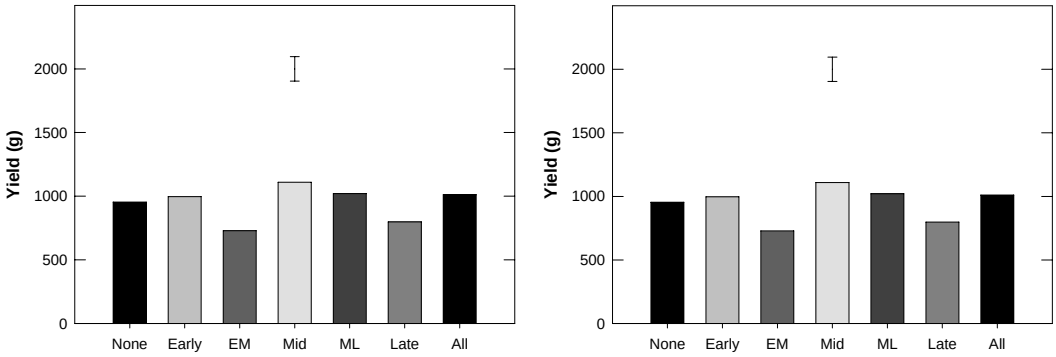


Figure 4.6 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Baldwin treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2006 using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

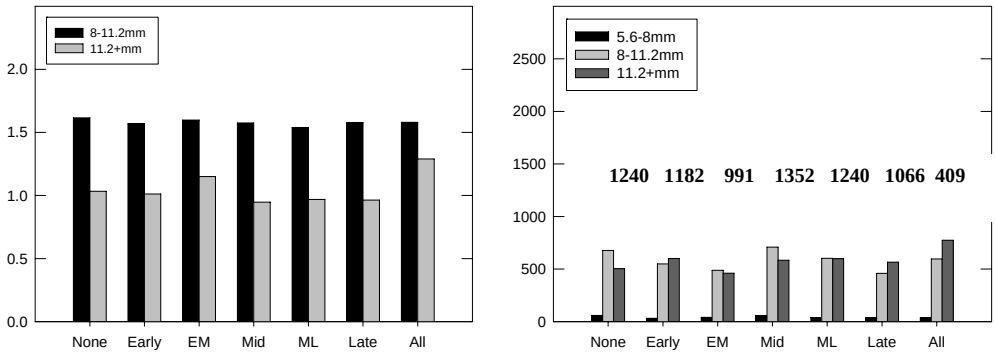


Figure 4.7 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Baldwin treated with Farm-Fos-44 [32% P_2O_5 and mean figures for total yield of AsA bush^{-1} treatment^{-1} (g AsA bush^{-1})

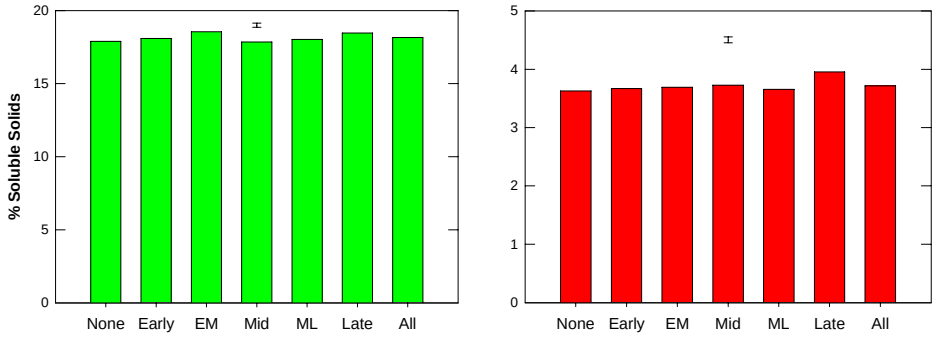


Figure 4.8 Mean fruit soluble solids (% Brix, left) and citric acid concentration (% w/w, right) from bushes of Baldwin treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2006 using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

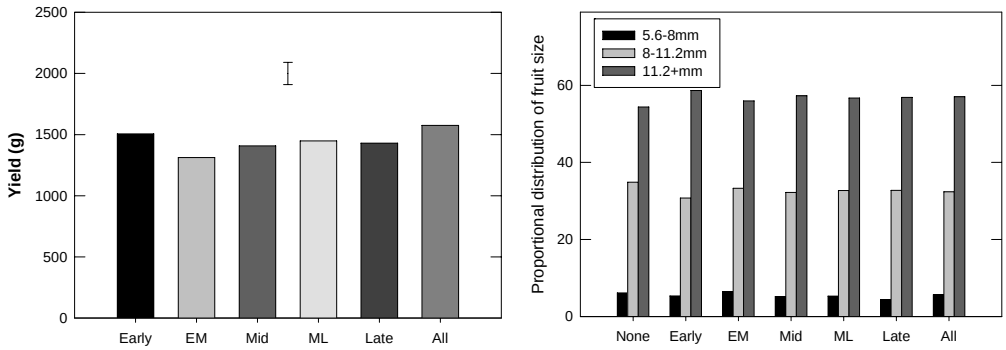


Figure 4.9 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Ben Lomond treated with Farm-Fos-44 [32%P_2O_5 and 29% K_2O] in 2006 using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

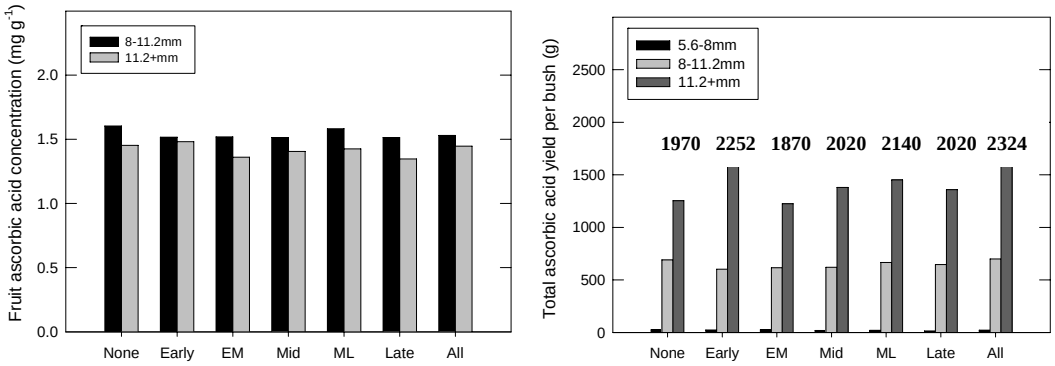


Figure 4.10 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Ben Lomond treated with Farm-Fos-44 [32%P_2O_5 and 29% K_2O] in 2006 using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}. Also shown are mean figures for total yield of AsA bush^{-1} treatment^{-1} (g AsA bush^{-1})

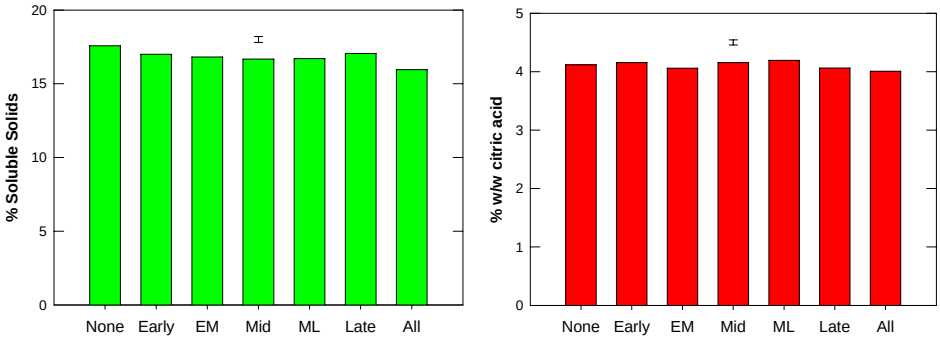


Figure 4.11 Mean fruit soluble solids (% Brix, left) and citric acid concentration (% w/w, right) from bushes of Ben Lomond treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2006 using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

Table 4.2 Statistical analysis of Baldwin and Ben Lomond AsA data contained in Figs 4.6 and 4.9. Bushes were treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2006 using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

Baldwin	*F Probability*			SED		
AsA concentration	8-11	11+		8-11	11+	
	ns	0.023		0.062	0.107	
Baldwin	*F Probability*			SED		
Total AsA per bush	5.6-8	8-11	11+	5.6-8	8-11	11+
	ns	ns	ns	12.35	146.2	123.4
Ben Lomond	*F Probability*			SED		
AsA concentration	8-11	11+		8-11	11+	
	ns	ns		0.055	0.062	
Ben Lomond	*F Probability*			SED		
Total AsA per bush	5.6-8	8-11	11+	5.6-8	8-11	11+
	ns	ns	ns	7.77	110.2	218.5

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Carry-over effect of potassium phosphite applications on fruit [AsA]

Year 5 (2006)

There was an apparent general decline in the fruit yield of Baldwin associated with the application of Farm-Fos the previous year (2005), but this was not statistically significant (Fig. 4.12). There was no significant treatment effects on the proportional distribution of fruit size and no influence on fruit AsA concentration, or total yield of AsA per bush (Fig. 4.13). There were no treatment effects linked to soluble solids or citric acid concentration for Baldwin (Fig. 4.14).

For Ben Lomond bushes treated with Farm-Fos in 2005 there was no carry-over effects on fruit yield, proportional size distribution, fruit soluble solids or citric acid concentration (Figs 4.15 and 4.17). Equally, there was no treatment influence on fruit AsA concentration, or total yield of AsA per bush (Fig. 4.16). The details of the AsA statistical analysis are shown in Table 4.3.

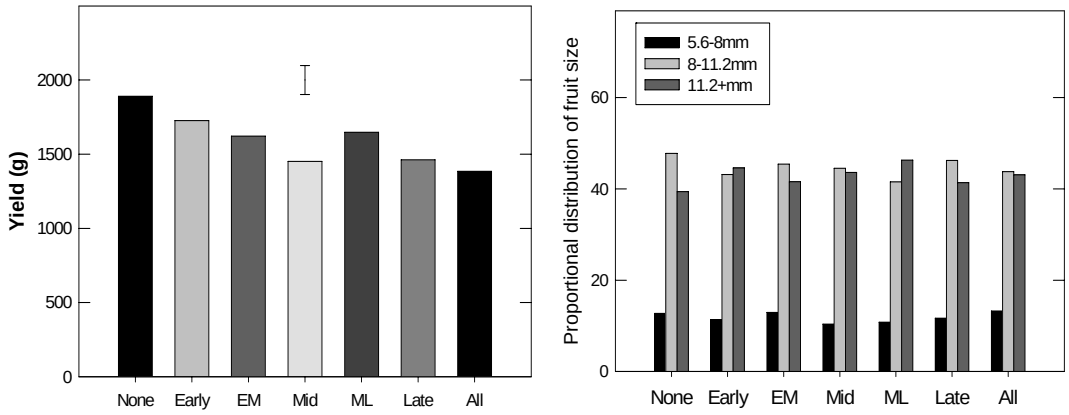


Figure 4.12 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Baldwin treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

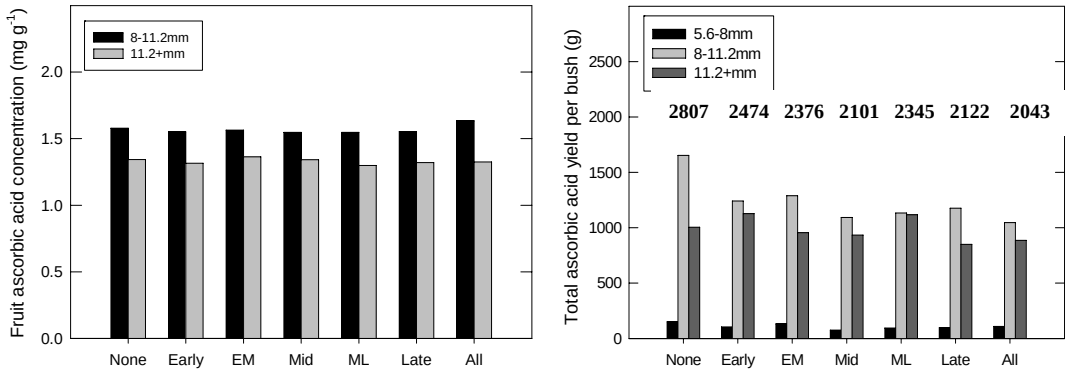


Figure 4.13 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Baldwin treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}. Also shown are the mean figures for total yield of AsA bush^{-1} treatment^{-1}

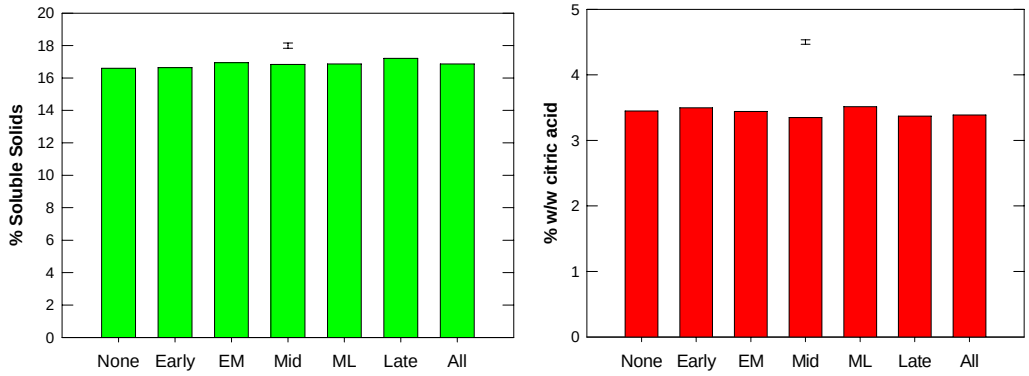


Figure 4.14 Mean fruit soluble solids (% Brix) and citric acid concentration (% w/w) from bushes of Baldwin treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

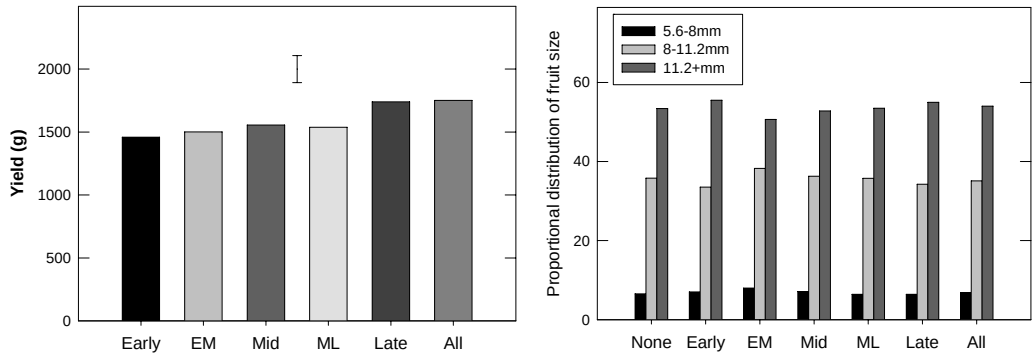


Figure 4.15 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Ben Lomond treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not in 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

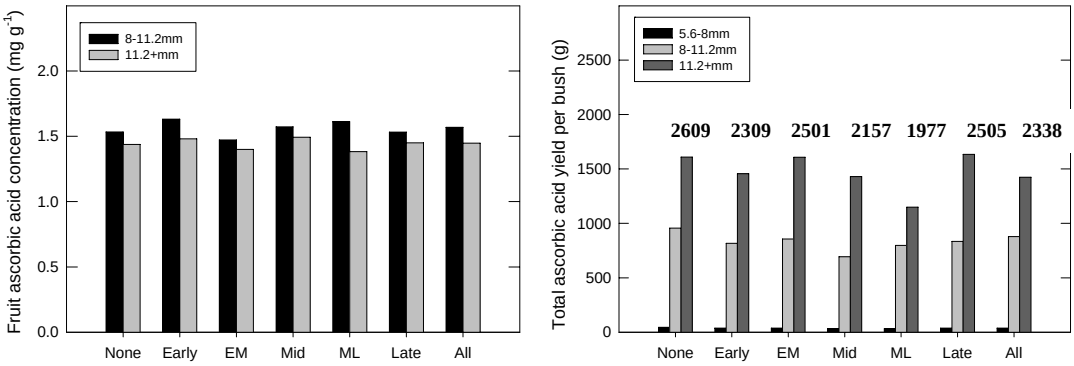


Figure 4.16 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Ben Lomond treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not in 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}. Also shown are the mean figures for total yield of AsA bush^{-1} treatment^{-1}

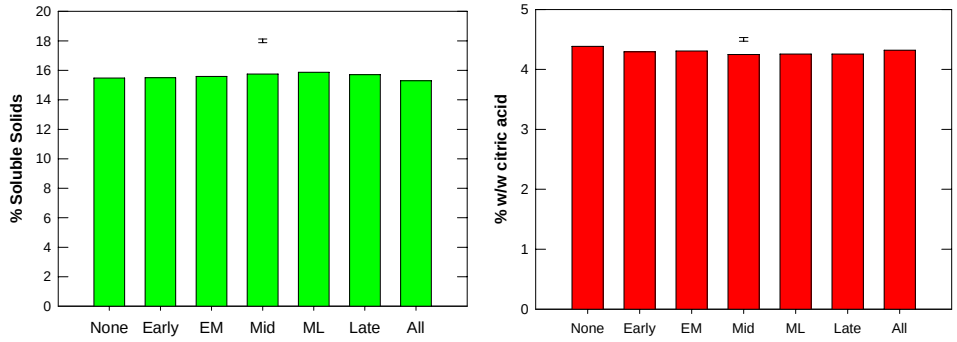


Figure 4.17 Mean fruit soluble solids (% Brix) and citric acid concentration (% w/w) from bushes of Ben Lomond treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

Table 4.3 Statistical analysis of Baldwin and Ben Lomond AsA data contained in Figs 5.2 and 5.5. Bushes were treated with Farm-Fos-44 [32% P_2O_5 and 29% K_2O] in 2005 but not 2006, using standard industry recommended rate of 1% (v/v), i.e. (10 litres of product) hectare^{-1}

Baldwin	*F Probability*			SED		
AsA concentration	8-11	11+		8-11	11+	
	ns	ns		0.0855	0.0857	
Baldwin	*F Probability*			SED		
Total AsA per bush	5.6-8	8-11	11+	5.6-8	8-11	11+
	0.019	0.011	ns	42.63	325.3	298.2
Ben Lomond	*F Probability*			SED		
AsA concentration	8-11	11+		8-11	11+	
	0.060	ns		0.051	0.121	
Ben Lomond	*F Probability*			SED		
Total AsA per bush	5.6-8	8-11	11+	5.6-8	8-11	11+
	ns	ns	ns	9.55	129.6	240.1

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Objective 5 - Develop strategies for maximisation of AsA production

Rationale

The primary aim of objective 5 was to combine successful treatments used in objectives 3 and 4 to develop a grower blueprint for maximisation of fruit AsA.

Headlines

- Combined irrigation and mulch had significant positive impacts on fruit yield

Multi-application treatment – irrigation and reflective mulch

Year 5 (2006)

In the first year of this combination experiment in which irrigation and mulching were applied together neither Baldwin or Ben Lomond showed a large fruit yield response to these treatments (Figs 5.1 and 5.4). This is perhaps surprising considering that this was a particularly dry year. However, there was a trend for fruit yield to increase with irrigation on Ben Lomond, irrespective of mulch (Fig. 5.4). The benefits of irrigation was also apparent, with Ben Lomond, when looking a fruit size distribution, where the application of irrigation reduced the proportion of smaller sized berries and increased those within the larger fruit size category (Fig. 5.4).

The impacts of mulching and irrigation on Baldwin and Ben Lomond fruit [AsA] were small with few statistically significant responses which could be linked to treatment application (Figs 5.2 and 5.5). There was, however, a significant reduction in AsA concentration for Baldwin fruit in the larger size category (11.2mm+). This was most likely due to the impact of irrigation on fruit expansion.

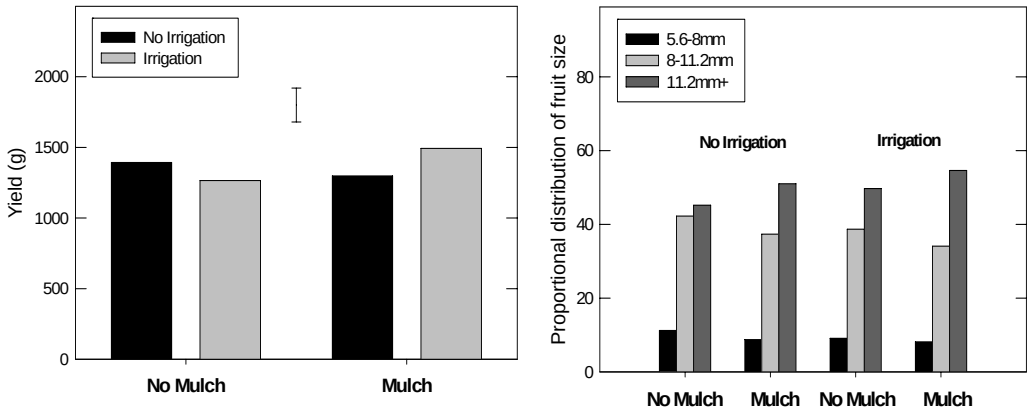


Figure 5.1 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Baldwin grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

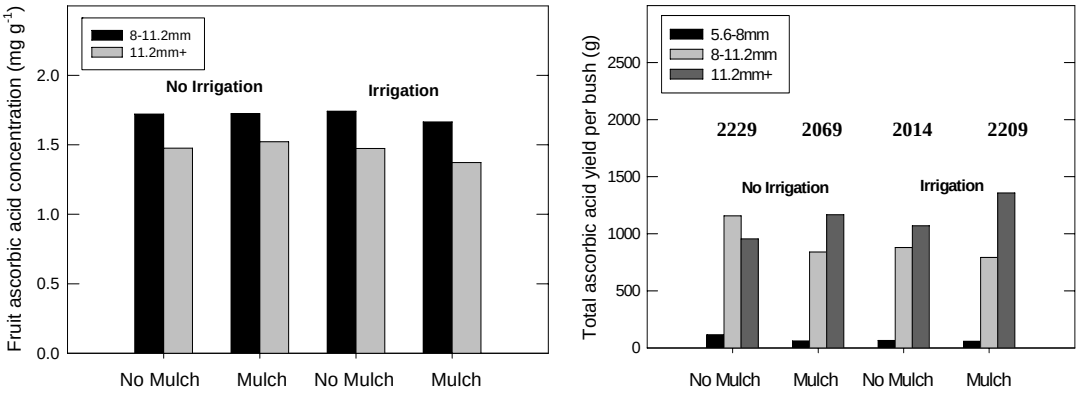


Figure 5.2 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Baldwin grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

Total fruit AsA yield per berry size, for both cultivars, showed some impact of irrigation, in particular Ben Lomond, with irrigated bushes having greater proportion of their total yield of AsA within the larger berry size category (Figs 5.2 and 5.5). Also of interest was the result that irrigation of Ben Lomond showed a positive trend of increasing total AsA bush yield despite not being statistically significant (Fig. 5.5). The carry-over impacts of

these treatments and their repeated application in 2007 may reveal further benefits to AsA yield.

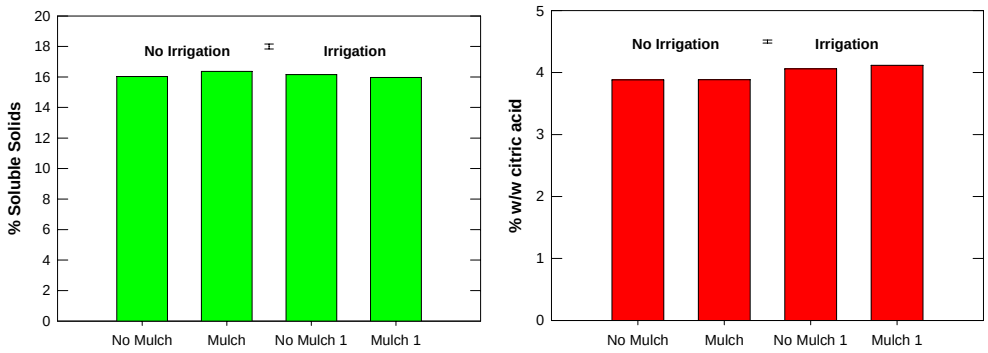


Figure 5.3 Mean fruit soluble solids (% Brix) and citric acid concentration (% w/w) from bushes of Baldwin grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

Table 5.1 Statistical analysis of Baldwin AsA data contained in Fig. 5.2. Baldwin bushes were grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

Fruit AsA concentration	*F Probability*		SED	
Treatment	8-11	11.2+	8-11	11.2+
Irrigation	ns	ns	0.0387	0.0349
Mulch	ns	0.035	0.0387	0.0349
Irrigation and mulch	ns	0.041	0.0548	0.0493

Total AsA yield per bush	*F Probability*			SED		
Treatment	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Irrigation	ns	ns	ns	19.2	143.9	148.5
Mulch	ns	ns	ns	19.2	143.9	148.5
Irrigation and mulch	ns	ns	ns	27.15	203.4	210.0

Treatment	*Total AsA per bush*	Treatment	*F Prob*	SED
NM NI	2229	Irrigation	ns	548
M NI	2069	Mulch	ns	548
NM I	2014	Irrigation and mulch	ns	775
M I	2209			

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

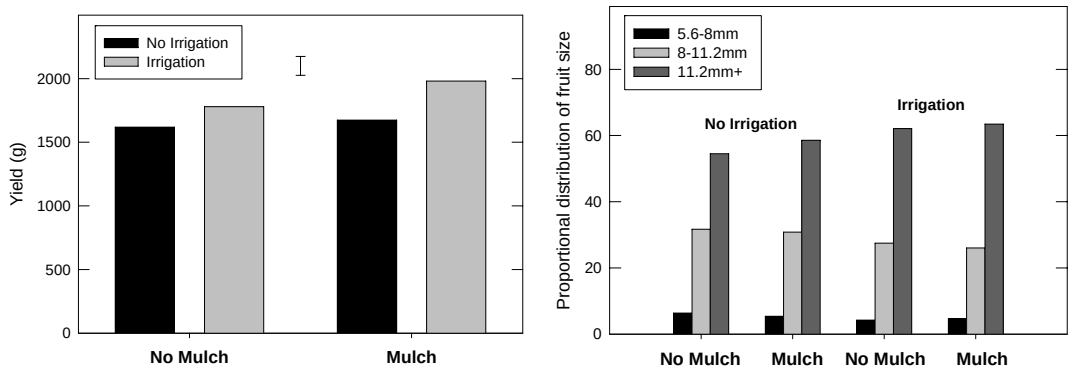


Figure 5.4 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Ben Lomond grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

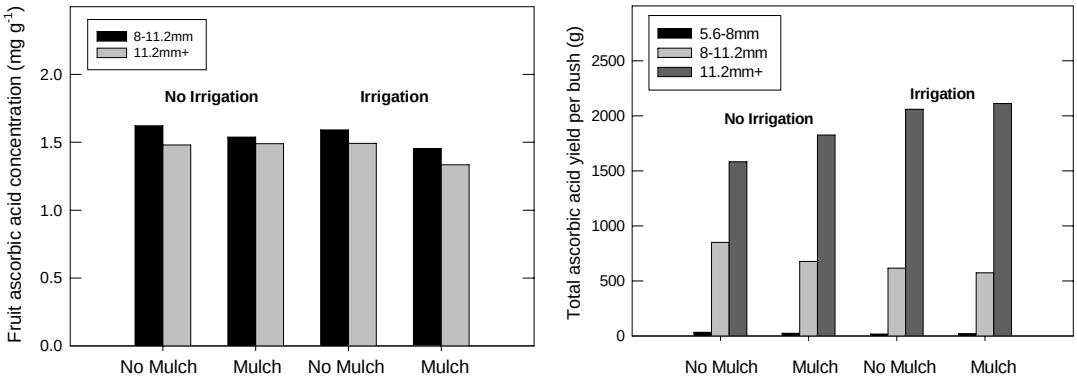


Figure 5.5 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Ben Lomond grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

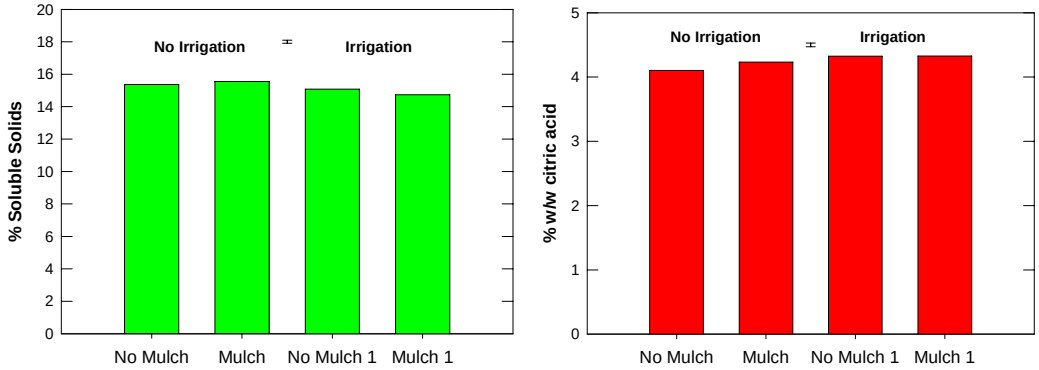


Figure 5.6 Mean fruit soluble solids (% Brix) and citric acid concentration (% w/w) from bushes of Ben Lomond grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season.

Table 5.2 Statistical analysis of Ben Lomond AsA data contained in Fig. 5.5. Bushes were grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

Fruit AsA concentration	*F. Prob*			SED		
Treatment	8-11		11.2+	8-11		11.2+
Irrigation	0.016		0.067	0.0439		0.0399
Mulch	ns		0.079	0.0439		0.0399
Irrigation and mulch	ns		0.044	0.0621		0.0564
Total AsA yield per bush	*F. Prob*			SED		
Treatment	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Irrigation	ns	ns	ns	3.88	67.9	135.1
Mulch	0.019	0.017	0.007	3.88	67.9	135.1
Irrigation and mulch	ns	ns	ns	5.48	96.7	191.0
Treatment	*Total AsA per bush*		Treatment	*F. Prob*		SED
NM NI	2467		Irrigation	ns		362
M NI	2527		Mulch	ns		362
NM I	2695		Irrigation and mulch	ns		512
M I	2708					

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Year 6 (2007)

Again as apparent in the previous year (2006) the influence of light reflective soil mulch was not particularly large but there was a small increase in yield particularly for Ben Lomond bushes that were mulched and irrigated (Fig. 5.7). However, there were no consistent treatment effects on fruit size distribution or fruit [AsA], irrespective of berry size (Fig. 5.8). The pattern of treatment response was similar with Baldwin but fruit yield differences were more significant (Fig. 5.9). With Baldwin the irrigation response was small, as might be expected in what was a wet year, but the reflective mulch produced a significant increase in yield, particularly when in the presence of irrigation (Fig. 5.9). This difference in yield was not reflected in specific changes in the proportional distribution of fruit to different size classes. However, the despite no dramatic change in fruit [AsA] the total yield of AsA per bush increased with irrigation and the use of reflective mulches (Fig. 5.10).

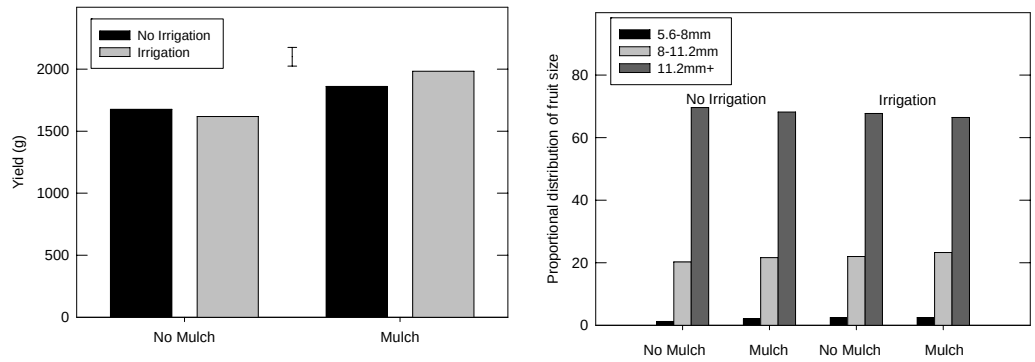


Figure 5.7 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Ben Lomond grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

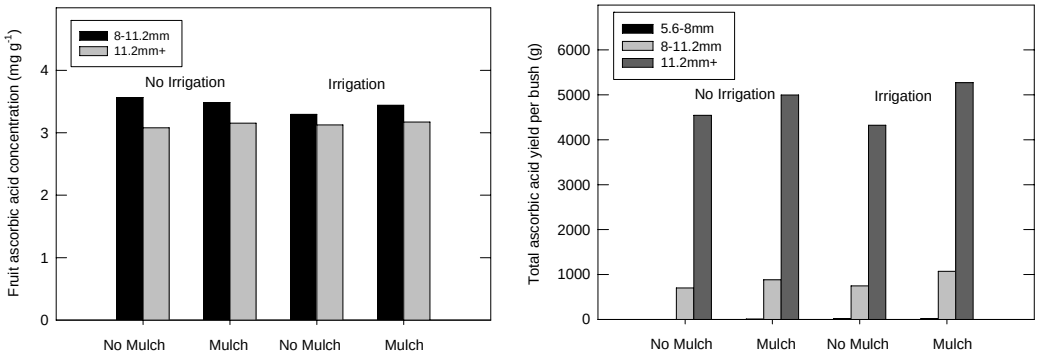


Figure 5.8 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Ben Lomond grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

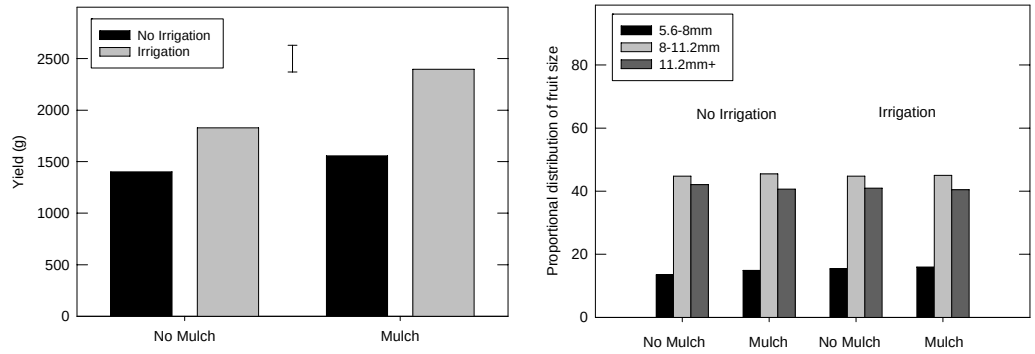


Figure 5.9 Mean fruit yields (left) and proportional (%) fruit size distribution (right) per bush of Baldwin grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

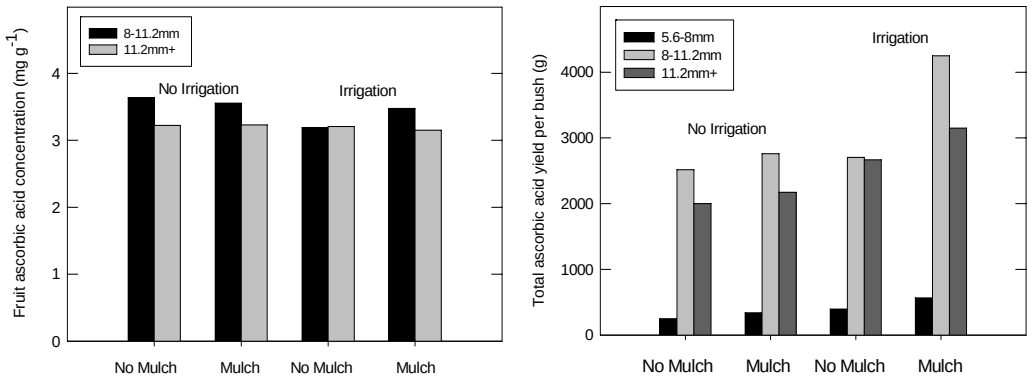


Figure 5.10 Mean fruit ASA concentration (left) and total ASA yield per bush (right) of Baldwin grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

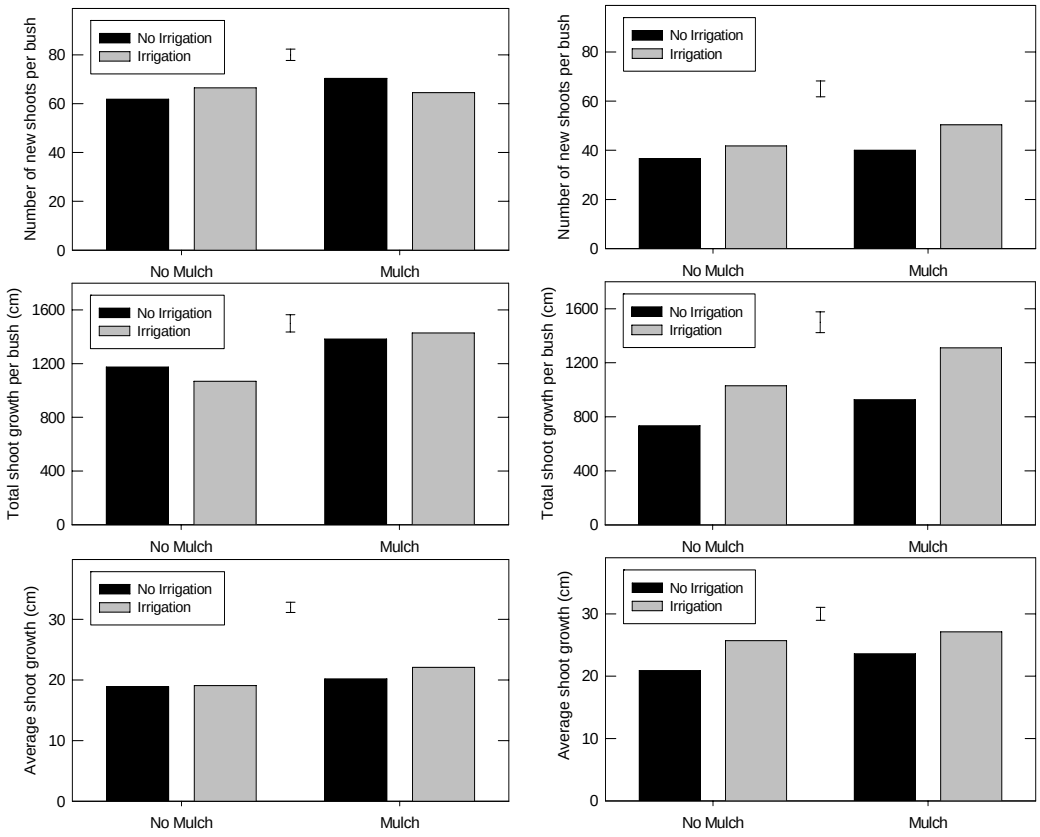


Figure 5.11 Number of new shoots, total shoot growth and the average shoot growth per shoot for bushes of Ben Lomond (left) and Baldwin (right) grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

The beneficial influence of irrigation and light reflective mulches of fruit yield of Baldwin appear to at least be partially associated with increases in vegetative growth (Fig. 5.11).

Table 5.3 Statistical analysis of Ben Lomond AsA data contained in Fig. 5.8. Bushes were grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

Fruit AsA concentration	*F. Prob*		SED	
Treatment	8-11	11.2+	8-11	11.2+
Irrigation	ns	ns	0.15	0.06
Mulch	ns	ns	0.15	0.06
Irrigation and mulch	ns	ns	0.21	0.09

Total AsA yield per bush	*F. Prob*			SED		
Treatment	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Irrigation	ns	ns	ns	4.4	90	311
Mulch	ns	0.008	0.03	4.4	90	311
Irrigation and mulch	ns	ns	ns	6.2	127	440

Treatment	*Total AsA per bush*	Treatment	*F. Prob*	SED
NM NI	5249	Irrigation	ns	358
M NI	5889	Mulch	0.01	358
NM I	5087	Irrigation and mulch	ns	507
M I	6361			

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

Table 5.4 Statistical analysis of Baldwin AsA data contained in Fig. 5.10. Baldwin bushes were grown as follows: no mulch and no irrigation (NMNI control), no mulch with irrigation (NMI), mulch without irrigation (MNI) and mulch plus irrigation (MI). Treatments were applied throughout the growing season

Fruit AsA concentration	*F Probability*			SED		
Treatment	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Irrigation	ns	ns	ns	0.09	0.16	0.06
Mulch	ns	ns	ns	0.09	0.16	0.06
Irrigation and mulch	ns	ns	ns	0.13	0.23	0.09

Total AsA yield per bush	*F Probability*			SED		
Treatment	5.6-8	8-11	11.2+	5.6-8	8-11	11.2+
Irrigation	<0.001	0.024	0.012	47.5	356.8	313.1
Mulch	0.009	0.016	ns	47.5	356.8	313.1
Irrigation and mulch	ns	ns	ns	67.2	504.7	442.7

Treatment	*Total AsA per bush*	Treatment	*F Prob*	SED
NM NI	4770	Irrigation	0.005	616
M NI	5274	Mulch	0.034	616
NM I	5766	Irrigation and mulch	ns	871
M I	7965			

Note: SED is Standard Error of Differences between the means, d.f. is residual degrees of freedom for comparison P is probability

CONCLUDING DISCUSSION

Mechanism and control of AsA accumulation in blackcurrant fruit

Work undertaken under objective 1 clearly suggested that ascorbic acid accumulation occurred in fruit as a result of *in situ* biosynthesis via the L-galactose pathway. Accumulation ceased at an early stage in fruit development as a result of decreased biosynthetic capacity and enhanced turnover despite the large excess of substrate (sugars) available for AsA synthesis. These data suggested that AsA accumulation was under tight developmental control and suggested that agronomic practices designed to enhance the supply of sugars further, particularly in early stages of fruit development might enhance fruit AsA concentration. It was therefore recommended that experiments should be undertaken to enhance light availability within the plant canopy and to develop treatments to encourage a strong, early flush of leaf growth.

Several experiments suggested some of the potential problems that may be encountered in trying to agronomically manipulate fruit AsA concentration. These included the finding that postharvest defoliation had little impact on fruit quality parameters (including AsA concentration) in the following year but reduced yield by up to 50%, the finding that following the removal of a treatment to artificially enhance fruit AsA concentration levels rapidly fell back to those of untreated fruit and the year to year hierarchy in fruit AsA concentration maintained across different blackcurrant cultivars. This data strongly suggested a tight genetic control of fruit AsA concentration and demonstrated both the feasibility and the necessity for the development of markers to allow the enhanced breeding of high AsA cultivars.

Agronomic approaches to the enhancement of fruit AsA

The overall aim of this ambitious five-year project was to highlight practical strategies that could be subsequently developed to ensure that growers were able to deliver fruit of a quality that facilitated the needs of juice manufacturing process. The key, but not only objective, being to achieve enhanced concentrations of AsA in berries. With respect to the agronomic aspects this was to be achieved by initially developing an understanding of the biosynthetic process which potentially could lend them to being manipulated in field-based

growing systems. Implicit was that such agronomic manipulations would be both potentially practically and economically viable for the industry to subsequently develop. At the outset very little was known in the published literature even about the more fundamental aspects of AsA biosynthesis in fruits, let alone what aspects might be manipulated in commercial plantations. Therefore, a series of pot-based experiments were carried out to explore and develop the potential approaches that could be used to manipulate berry [AsA]. These initial approaches included manipulation of plant nitrogen supply and water availability. Both these resources are known to influence plant growth and development and in some cases the accumulation of phytochemicals such as AsA. This is because the synthesis of many plant-based phytochemicals, including ascorbic acid (vitamin C or AsA), is induced by environmental stress. Such that, for example, drought may enhance biosynthesis, while optimal leaf concentrations of nitrogen may have the opposite effect. On this premise an extensive series of experiments were undertaken using field-based experiments. The opportunities to increase radiation levels within the crop, via the use of light reflective mulches, were also seen as a possible route by which the plant's innate responses to light-stress might enhance [AsA]. Work was also undertaken to ascertain a more fundamental insight into the internal physiological growth and development processes that might impact on [AsA]. To achieve this, experiments were undertaken to determine how the level of yield influenced fruit [AsA] along with the 'source:sink' relationships within the plant that are known to clearly influence fruit growth and development in other crops.

Manipulating nitrogen supply

Initial pot-based experiments with cvs Hedda and Baldwin showed a very clear response to increases in applied nitrogen. Destructive leaf tissue analysis confirmed that nitrogen uptake changed with substrate nitrogen availability up to an asymptotic level. Field-based studies showed that at a supply rate greater than 152 kg N ha^{-1} there was little further agronomic value (particularly yield gain) of adding more nitrogen.

An over-abundant nitrogen supply can lead to excessive vegetative growth, which could shade fruit and thereby reduce fruit AsA concentration (Lee & Kader, 2000; Ma & Cheng, 2003).

From a practical sense it was also established that direct measurements of leaf chlorophyll content could not only be used as a proxy for direct measurements of leaf protein concentration, but also as clear non-invasive indicator of differences in plant nitrogen uptake.

Using nitrogen at 'excessive' levels promoted bush growth, particularly leaf canopy development. Fruit berry size also increased with bush nitrogen status. But this appeared to be at the expense of fruit [AsA] which declined with increasing nitrogen. An explanation of this may be that increased shoot and leaf production may cause an increased shading of leaves close to the developing fruit (reduced photosynthesis), as well as shading the fruit itself. This may reduce the carbohydrate available for fruit growth and or the substrates for AsA biosynthesis.

The cv. Hedda consistently showed a lower berry [AsA] than cv. Baldwin, as expected (Viola *et al.*, 2000), with Baldwin yielding an approximately two-fold greater concentration. However, both cvs respond in a similar manner to increasing nitrogen concentration. This supports the notion that agronomic methods can be used to enhance [AsA] in both high AsA, as well as, in low AsA cultivars.

In field-plantings it was more difficult to demonstrate the value of not over using nitrogen. This was probably because the general field soil levels at EMR were greater than the optimum required to maximise plant nitrogen concentration (protein status). Longer-term field experimentation (Year 4, 2005) was beginning to show signs of nitrogen deficiency in the larger bushes of Baldwin which had received suboptimal nitrogen fertilisation for at least two years previously.

Again as with the earlier pot experiments excess nitrogen applied in the field had a small but significant negative impact on fruit [AsA] as well as total yield of AsA.

Manipulation of crop load

It can be hypothesised that if AsA biosynthesis and export occurs solely from leaves (Franceschi & Tarlyn, 2002) then altering the fruit to leaf area ratio should impact on fruit AsA concentration. To initially test this hypothesis the amount of crop (fruit yield) was manipulated by the removal of whole flowering strigs from potted plants of Baldwin and Hedda.

When crop levels were adjusted early in the fruit development cycle (at flowering) as would be expected there was a proportional reduction in yield in direct response to the level of strig removal, irrespective of cultivar. There was some suggestion that the cv. Baldwin compensated to a greater extent, with respect to increased fruit size, when crop load per bush was reduced compared with Hedda (see Toldam-Andersen & Hansen, 1993). However, this reduction in crop load had no influence on fruit [AsA].

An experiment was carried out with Baldwin plants to which all the strigs on some plants had their fruit number reduced by around 50%. Again, Baldwin showed a capacity to offset the reduction in potential yield (only 22% reduction) in the 50% fruit reduction treatment by increasing the size of the remaining berries within a strig. This reduction in strig fruit number did not however significantly change the berry [AsA].

A further experiment was carried out with Ben Lomond and Baldwin field-grown plants to which, again, 50% of the strig berry number were removed earlier in the fruit development cycle. Fruit removal during earlier development when [AsA] is at it highest is hypothesised to have the likelihood of the greatest impact; if the fruit are not the source of AsA and it fruit sink competition exists. Again the cv. Baldwin showed the greater ability to compensate for a reduction in fruit strig number by increasing berry size, particularly with respect to largest size berries within a strig. There was again, however, no significant change to the berry [AsA].

These results support the notion that there appears to be no obvious competition between berries within a strig for an externally supplied source (leaf-derived) of AsA.

Natural variation in strig berry number was also considered as a means by which regulatory activity on AsA production and berry concentration could be measured. Much of this variation in strig berry number is likely to be due to the abscission process described as ‘run-off’. To evaluate this hypothesis a comparative descriptive study of ‘large’ (8-14 berries) and ‘small’ (2-5 berries) strigs was undertaken. Mean berry sizes between strigs were similar (~480mg). However, the [AsA] was highest in all berry sizes on the smaller strigs, but total AsA content was greater in the larger strigs (19mg compared to 7mg of AsA) due to their larger number of berries. Therefore yield is the overriding factor determining total bush yield of AsA.

A further experiment was carried out to manipulate strig leaf area and the availability of carbohydrates from other part of the bush. The latter was achieved by 'ring girdling' the phloem at different locations, while the former involved removal of the leaves associated with a particular strig. Strig leaf removal was clearly linked to a reduction in fruit fresh weight and fruit number. Girdling branches also reduced fruit yields. A combination of strig leaf removal plus branch girdling reduced fruit yields even more. The results of this experiment showed that around 50% of the carbohydrate for fruit growth came from leaves at the tip of the shoot, while between 20 to 30% came from the strig leaves and the remainder came from 'storage' (in the woody tissues, stems and roots). The impacts of this manipulation of fruit carbohydrate source on [AsA] did not clearly support the notion that AsA biosynthesis was directly, or solely, dependent on strig leaf photoassimilates or stored carbohydrates.

Manipulation of water supply

An initial pot-based experiment attempted to provide plants with irrigation to only part of their root system. The approach of partial root irrigation has in many cases enabled a restrictive control treatment to be applied to vegetative plant growth. The hypothesis was that combined with a reduction in shoot growth there may be beneficial redirection (change in 'sink') of photoassimilates towards reproductive growth investment (fruit production) apparent as enhancement of phytochemicals, such as AsA.

An experimental approach to restrict pot water availability, to part of the plant's root system, was carried out during different stages of fruit development. Despite cultivar differences in dry matter allocation to different plant parts there was no evidence that withholding water had reduced vegetative shoot growth. The impact of drought on fruit [AsA] was generally not consistent between all treatments or size of berries.

An impact of drought related to differences in berry size was expected, since previous work (Viola et al., 2000) has shown that while the amount of AsA per berry remains relatively constant during blackcurrant fruit development, the berry [AsA] declines as the berries swell during ripening. Thus if drought reduced berry size an increase in berry [AsA] might be expected.

A similar approach to the experiment was used to withhold water from plants growing in a field-planting using double irrigation lines down a row of bushes, to water alternate sides of the bushes roots under a black polythene mulch (to exclude precipitation). Five irrigation regimes were set up to include, plus and minus irrigation, no plastic mulch, and irrigation on either side on the row (east and west). Soil moisture monitoring, throughout the season, showed that targeted differences in soil moisture were achieved, and these impacted on vegetative shoot growth. Bushes not receiving full irrigation had reduced shoot growth. There were no negative effects of the restriction in water availability on fruit yield per bush, but berry size was influenced.

A continuation experiment was carried out in the following year comparing no irrigation with partial and full irrigation. Despite some difficulties in establishing large treatment differences in soil moisture content, a comparison between Ben Lomond and Baldwin showed that the amount of water used to irrigate these field-crops impacted positively on Baldwin fruit yield. There was however, no impact on berry [AsA], but a large positive enhancement on total AsA yield per bush.

Manipulation of light interception

Enhanced interception of solar radiation, by leaves, benefits plant growth and crop yield directly by driving the photosynthetic process it may also enhance photosynthetic processes going on in fruit particularly during early development when still green. However, other regions of the solar spectrum may also contribute to influencing both plant morphological and biochemical biosynthetic process. For example, the ultra-violet regions of the solar spectrum are known to be linked with stress development and synthesis of plant pigments and antioxidants such as AsA.

The aim was to evaluate an approach to maximise bush solar radiation interception by enhancing the use of reflected radiation. This was achieved by placing various types of reflective polythene mulches on the soil directly below the leaf canopy and to some extent into the bush alleyways of field-grown bushes. The effectiveness with which these materials were used to enhance, or recycle solar radiation back into the leaf canopy was clearly quantified and significantly different from bare soil. Environmental monitoring showed that the influence of the mulch was primarily confined to radiation responses, not air, or soil temperature changes induced by the mulch.

Reflective mulches increased the canopy growth of bushes by significant amounts within a growing season and this was accompanied by a relatively small but not significant increase in fruit yield. There was no influence on fruit [AsA], or total bush yield of AsA.

The field-based experiments using light reflective mulches were continued into a further growing season to determine the impact of the increased shoot growth from the previous season and to look at the impact of accumulative factors. Again, there were small increases in yield linked to the reflective mulch treatments applied to both Ben Lomond and Baldwin, but no change in fruit [AsA] or bush yield.

Developing strategies for maximisation of [AsA] production in the field

A number of field-based experiments were carried out throughout this project to evaluate commercially practical approaches to enhancing fruit [AsA]. These included the possibility that precursors of the AsA biosynthetic process might limit AsA production. Applications both from field and laboratory-based experiments failed to suggest that the application of the immediate precursor to AsA, i.e. L-galactono-1,4-lactone enhanced the [AsA] in fruit. It should be mentioned that chemical analysis to prove that the precursor was taken up during the treatment and in an appropriate cellular location and state for incorporation into AsA was not undertaken. The practical aims of the approach defined that the response, or lack of it, was of key importance rather than definitive proof of the reason for a negative response.

Field-based approaches were also used to manipulate the nutritional constituents of plant tissues and fruits The aims of this work were driven primarily by anecdotal evidence from industry partners and discussions at various consortium meetings. The approach has relied primarily initially on field-based 'trials', in many cases not as statistically rigours as the standards used in designed field experimentation at East Malling Research. The reasons for this are that they have been carried out by growers and applied on grower farms with mature existing plantings, using standard commercial on-farm operations and application technologies. None of these 'nutritional supplement' approaches (phosphorus, potassium and calcium) have revealed any significant positive influences on fruit [AsA] from these initial trials. This is despite some suggestions from the literature to the contrary (Bould 1968). The same result was true where duplicated similar statistical designed trials were carried out at EMR.

Multi-application treatment – irrigation and reflective mulch

Initial result from field-based experiments to determine the beneficial impacts of different combinations of treatments did not produce short-term positive effects. This was not perhaps surprising given yearly results which had implied how 'tightly' AsA biosynthesis appears to be regulated. The effects that were reported were generally small and always linked to small enhancements of fruit yield. Factors such as the value of irrigation are however only likely to be clearly beneficial in years when natural precipitation is low.

We have hypothesised throughout this work that the culmination of the project in developing new strategies for growers would not only require the likely benefits gained from additive beneficial treatment responses but also those from cumulative benefits gained over time. This has been particularly apparent for the results obtained in 2007, for field experiments, where treatments have been in place and reapplied over more than one growing season.

The combination of irrigation and light reflective mulches, in field planting, can produce significant positive enhancements to fruit yield and the total yield of AsA per bush of Baldwin. These benefits have increased with time and may increase further.

Fruit [AsA] has been shown to be closely regulated, irrespective of berry size, fruit nutrition, crop load and the proportional distribution of fruit to various size classes.

All of the agronomic approaches have produced increases in fruit yield many of which have been statistically significant and this factor has contributed to significantly important enhancement in the bush AsA yield.

Development of markers for the rapid selection of high AsA cultivars

Both the biochemical/physiological work and the applied agronomic work have shown the need to breed high AsA cultivars. Although genetic material currently exists within breeding programs that contains exceptionally high levels of AsA (up to 500 mg / 100 ml juice), the genotypes fail to meet other important agronomic, physiological or quality criteria. The development of genetic markers to allow the determination of blackcurrant genotypes containing acceptable levels of AsA at a pre-fruiting stage will allow increased

number of crosses to be performed in each year thereby facilitating the development of novel cultivars containing acceptable levels of AsA combined with other essential traits.

EXPLOITATION PLAN

Future R&D resulting from the project

Having identified the source of variability in fruit AsA concentration (biosynthetic capacity) as part of the LINK and shown how this is linked to genetic variation within GDP-mannose epimerase, further work will be undertaken to seek additional molecular markers and develop appropriate deployment strategies. The following specific actions are planned:

- Obtain full length clones of the genes encoding four crucial AsA biosynthetic enzyme activities (GDP-mannose pyrophosphorylase, GDP-mannose 3,5-epimerase, GDP-L-galactose guanyltransferase and L-galactose-1-phosphate phosphatase).
- Compare coding sequences of the four genes between high and low AsA genotypes to identify variants associated with high fruit AsA (as achieved for the epimerase).
- Conduct bioinformatic analysis of sequence variants to determine likely impact of variation on enzyme biochemical properties.
- Perform kinetic characterisation of heterologously expressed proteins to confirm bioinformatic findings.
- Validate markers through crossing and segregation experiments.
- Deploy markers in selection strategies for breeding germplasm

Several sources of funding have been identified. These include a GSK-BBSRC CASE studentship to be undertaken at SCRI with the student registered at Dundee University and beginning in September 2008. Alternatively, Dr. Robert Hancock (SCRI) has been approached by a Chinese doctoral candidate who wishes to conduct the work under a China Scholarship Council funded scheme and additional funding will be sought (British Council, Great Britain-China Educational Trust, GSK, HDC, EU). Any additional follow-up will take place as part of Scottish Government commissioned research at SCRI and the GlaxoSmithKline (GSK) funded breeding programme. This work will provide GSK, the fruit industry and breeding programme managers with robust predictive molecular markers for use in blackcurrant breeding. It is estimated that the use of appropriate markers will accelerate the release of new cultivars with elevated vitamin C (associated with other desirable traits) by up to 5 years.

Further statistical analysis of historical records of fruit AsA concentration and climate is planned. Correlating these data against weather conditions, using multiple linear regression analysis, is envisaged as a means to clearly define the complex impact of multiple climate variables. This data will provide blackcurrant growers and GSK with i) short and long-term predictors for fruit AsA and ii) insights into optimal management of the blackcurrant crop. Data was collated and placed into a usable format as part of the LINK programme and it is envisioned that the work will be funded by GSK via a short-term (1-2 month) statistical consultancy undertaken in the financial year 2008-9.

Field experiments have highlighted a number of agronomic approaches that could be developed further to optimise fruit and therefore bush AsA yields:

- Work undertaken in the LINK programme demonstrated the importance of optimal nitrogen nutrition and this will be exploited by growers (see industrial relevance) however, further benefits may be accrued following further research. In particular, the impact of nitrogen application during early plant development on subsequent yield and AsA concentration and the response of different cultivars and growing systems to nitrogen application.
- The beneficial use of supplementary irrigation to enhance fruit yields was demonstrated even in wet years. However, in order to fully realise the potential of such an intervention a full cost-benefit analysis must be undertaken. This will require economic analysis in the context of predicted climate change and practical agronomic work to identify the most efficient means of irrigation both in terms of capital outlay and ongoing resource efficiency.
- Several lines of research demonstrated the importance of light interception on fruit yield. 1) Reflective mulches, particularly in conjunction with irrigation enhanced fruit yields by up to 15%; 2) Restriction of carbohydrate availability by defoliation had strong negative effects on fruit yield. Given the value of the blackcurrant crop, the area under cultivation and the fact that mulches would rapidly deteriorate as a result of machine harvesting the use of reflective mulches is not economically viable. Therefore, opportunities need to be developed to find commercially acceptable ways to maximise light harvesting by blackcurrant bushes. This could be achieved by developing pruning regimes to maximise light harvesting or by developing methods to extend the period to autumn leaf fall.

We envisage that the work outlined above will now be funded via the HDC levy fund specifically set aside for the blackcurrant industry. This fund is managed by the Blackcurrant Grower's Association. The blackcurrant growers R&D committee will meet on the 10th December 2007 and future research arising from the LINK programme is tabled for discussion. The final results of this work will also be presented at GSK Technical Meeting on March 12th 2008. This opportunity will be used to facilitate which aspects of the research will be supported by both GSK and levy funds.

Industrial relevance and plans for future commercial exploitation

UK growers produce in the region of 14,000 tonnes of blackcurrants with the vast majority destined for the processing market. The competitiveness of the UK industry is supported by processor investment in blackcurrant breeding which produces 1 - 2 novel cultivars each year, many of which are exclusively available to UK growers. There is also government support for underlying science research in the genetics and quality aspects of *Ribes*, with information deployed in the breeding programme. A new generation of cultivars can take up to 18 years from initial crossing to first farm production. Breeding has a number of objectives for future blackcurrant development, including enhanced flavour and colour, high juice yield, increased pest and disease resistance, and enhanced AsA. In order to remain competitive, UK blackcurrant growers need cultivars that combine both quality (AsA, flavour, colour, juice yield) and agronomic traits (environmental adaptability, P&D resistance). Molecular markers from this project will allow rapid development of cultivars combining these traits, thus maintaining the competiveness of UK growers through the capacity to supply the processing industry (value £200M per annum) with high quality fruit specifically tailored to their requirements. The availability of high AsA varieties represents a unique opportunity for processors to avoid AsA fortification, resulting in reduced production costs and increased potential consumer uptake. It is intended that variation identified in the gene encoding GDP-mannose epimerase will be exploited as soon as possible within the germplasm improvement programmes at SCRI, with immediate implementation of marker validation leading to full marker exploitation within 6 – 8 years. Furthermore, it is intended that further markers will be identified and implemented as a result of future R&D directly arising from the LINK project.

Field experiments have highlighted a number of agronomic approaches that growers should consider in order to enhance their fruit yields without compromising fruit AsA

concentration. The field approaches evaluated were those that could be considered as commercially applicable for UK growers. Biosynthetic control of AsA fruit concentration, at least in blackcurrant, is tightly regulated which therefore precludes the opportunity for significant agronomic manipulation of fruit quality. However, the results have clearly shown that growers can adopt a number of cultural strategies to increase the yield efficiency of their crops at the bush level. Importantly, adopting such strategies will not impact fruit quality and in particular, there will be no reduction in AsA content per berry. This information has relevance to fruit growers both for juice production and will also influence those considering the fresh market, with a high health value product.

In general, nitrogen excess had a negative impact on fruit AsA concentration, although nitrogen fertilisation had little impact on fruit yields. Advice to growers is therefore to closely monitor nitrogen nutrition to avoid nitrogen oversupply. Such advice is likely to offer growers both material and labour efficiency gains, and will be disseminated both through field officers of the major processor (which contracts the majority of UK growers) and through grower dissemination events.

The research has highlighted the importance and complexity of potential interactions between crop yield, quality and climate. The blackcurrant industry has particular concerns in climatic effects on cropping, especially regarding 'climate change' and the effects of reduced winter chilling on cropping. Changing rainfall patterns are already influencing cropping, and this will probably increase as the blackcurrant growing regions of East Anglia and southern England become drier in the summer. Under present climatic conditions the economic value of supplementary irrigation for blackcurrant is unclear, although irrigation was shown to enhance fruit yields even in wet years. As the climate changes the impact of rainfall and the need for supplementary irrigation will increase and this will influence future crop/juice yields. The industry must therefore closely monitor effects of environmental change on cropping regions in sensitive water catchments, and consider the costs and benefits of supplementary irrigation as climatic conditions become drier.

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TECHNOLOGY TRANSFER

A primary consideration for the project has been a clear and strong technology transfer strategy combined with efforts to publicise the research as widely as possible. Details of the project have been mounted on the SCRI website since the project start (http://www.scri.ac.uk/research/qhn/phytochemicals/vitamincinblackcurrants) and a project flyer was produced at an early stage and made available to all interested parties (http://www.scri.ac.uk/scri/file/Staff/Rob%20Hancock/HortLink%20flyer%2005.pdf).
Technology transfer has been achieved on several levels:

1. Publication in refereed journals for the science community.
2. Dissemination to the scientific community at international conferences.
3. Publications aimed at the broader research and policy community (e.g. BBSRC business, Agriculture LINK, Plant It).
4. Publications in trade magazines aimed at the grower community.
5. Dissemination to growers at grower based conferences including regular GSK organised grower conferences.
6. Dissemination to the general public via the popular press, general interest websites and broadcast media.

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APPENDICES

APPENDIX 1 – Realignment of Horticulture LINK programme 003/02

The document outlined below arose as a result of discussions at a project management consortium meeting in light of the results obtained after three years. The document was put to all members of the consortium including funding bodies and was approved in full prior to any changes in the work programme.

Realignment of Horticulture Link MRS 002/03 – Development of Physiological and Agronomic Tools for Increasing L-Ascorbic Acid Yield from Blackcurrant Bushes

Key Results Years 1-3

1. Mechanism of AsA accumulation in blackcurrant fruit

Results obtained in the first three years of the project have allowed the generation of a model for AsA accumulation in blackcurrant fruit. AsA is accumulated in blackcurrant fruit during the early stages of fruit development (green stages) by synthesis within the fruit using imported sugars as a substrate. AsA accumulation ceases as the fruit start to take on red colouration as a result of decreased biosynthesis and increased turnover. Evidence suggests that cultivar differences in fruit AsA concentration at maturity are determined by the early fruit AsA biosynthetic capacity in different cultivars. Current data suggests that the source of sugars for AsA biosynthesis are storage carbohydrates that are hydrolysed and transported to fruit via the phloem. We have some evidence to suggest that post-harvest carbohydrate accumulation may impact on fruit AsA accumulation in the following year.

2. Genotypic flexibility of fruit AsA concentration

A number of results suggest that there is a strong genotypic influence on fruit AsA concentration. Firstly, analysis of historical data shows that although there can be large (up to 2-fold) year to year variations in fruit AsA concentration of the same cultivar grown at the same location, the cultivar hierarchy is maintained. Secondly, artificial increases of fruit AsA concentrations by supply of its immediate biochemical precursor to intact fruit attached to the bush are rapidly reversed once the precursor is removed.

3. Environmental influence on fruit AsA concentration

Individual agronomic manipulations such as changes in light quality, drought stress and fruit thinning while able to have significant impacts on fruit yield and hence bush AsA yield have little impact (<15%) at the level of fruit AsA concentration. However, historical data suggest that combined agronomic treatments may have greater impact as year to year AsA concentrations of a particular cultivar grown at a particular location can vary up to 100%.

The data obtained to date suggest that (i) post-harvest agronomic treatments may significantly affect fruit AsA concentration in the subsequent season (ii) combined agronomic manipulations could significantly affect fruit AsA concentration (iii) there is a strong genetic component to fruit AsA concentration.

Original Work Plan Years 4-5

The original work plan called for further biochemical studies similar to those undertaken in years 1-3 using high AsA genotypes identified in screens of segregating and mutagenised populations.

- Milestone 1.16 – Complete biochemical and physiological characterisation of AsA accumulating mutants (if available).
- Milestone 1.20 – Complete biochemical/physiological characterisation of new enhanced germplasm.

An additional investigation to provide insight into fruit AsA accumulation was the investigation of the effects of temperature.

- Milestone 3.9 – Complete investigation on the effect of temperature on AsA production in blackcurrant bushes.

Further work to provide greater understanding of the mechanism of fruit AsA accumulation would be the identification of predictive markers. This work would also add value to ongoing blackcurrant breeding programs.

- Milestone 2.11 – Identify robust correlative markers that can be used to predict berry AsA content in pre-fruiting stages.

Two further milestones were designed to provide growers with practical methods for maximising fruit AsA.

- Milestone 4.7 – Complete repeat experiments and analysis of 2 cultivars x most promising agronomic manipulations.
- Milestone 5.3 – Complete grower field trials of agronomic manipulations to enhance AsA production and analyse data.

All of the above experiments were designed in part to allow fulfilment of milestone 5.4

- Milestone 5.4 – Develop mechanistic understanding of AsA synthesis and distribution in *R. nigrum*

Revised Work Plan Years 4-5

Although a number of relatively high AsA individuals were identified in both the mutagenised (270 mg/100ml juice) and segregating population (450 mg/100 ml juice), no plants with exceptional fruit AsA were identified. The biochemical model developed to date shows the same mechanism for AsA accumulation in low, medium and high AsA genotypes suggesting a universal mechanism for AsA accumulation in blackcurrant fruit. It is therefore highly unlikely that repeating the biochemical work already undertaken on blackcurrant genotypes with even higher fruit AsA will add further information and it is proposed to delete milestones 1.16 and 1.20.

The controlled environment work to understand the effects of temperature on fruit AsA concentration may provide valuable insights into the mechanisms of AsA accumulation and furthermore will provide growers with useful information regarding the likely effects of climate change on an important fruit quality trait (i.e. AsA concentration). Milestone 3.9 will therefore be fulfilled as originally proposed.

Due to the strong genetic component in the determination of fruit AsA concentration, predictive markers are likely to be a highly valuable tool in the long term effort to maintain and increase fruit AsA concentrations. The advantages of predictive markers are that they

will allow selective removal of all plants with an unacceptably low predicted AsA concentration in fruit at an early stage, shortly after germination. This will allow blackcurrant breeders to increase the numbers of seeds germinated in any given year as a certain proportion will be rejected at an early stage prior to the requirement for planting out in the field. This in turn means that there is a greater likelihood of breeding future cultivars that have an acceptable fruit AsA concentration in combination with other desirable characteristics such as disease resistance, low winter chill requirements, acceptable colour, aroma or flavour characteristics. The result will be an accelerated breeding program bringing cultivars with high AsA in combination with other desirable traits to the grower in a reduced timeframe. Given the potential benefits of such markers, it is proposed to increase the emphasis placed on milestone 2.11 over the final two years of the programme.

Proposed Work 2006-2007

1. Continue to refine blackcurrant AsA accumulation model

Work in the remaining two seasons will focus on the source of carbohydrate for AsA biosynthesis in immature blackcurrant fruit. The current hypothesis is that at the time of AsA accumulation leaves are not sufficiently developed to be fully functioning photosynthetic tissues and therefore the source of sugars must be stored carbohydrates. This hypothesis will be tested using two complementary approaches:

I. Direct measurement of starch concentration in plant tissues throughout the growing cycle

Whole plants will be harvested at different points in the growth cycle and the dry matter distribution between stems, roots, leaves and buds/flowers/fruit determined. The amount of sugars and starch in each tissue will be estimated using standard procedures developed at SCRI (Viola and Davies, 1992) and the annual carbohydrate balance calculated. If starch reserves are the ultimate substrate for fruit AsA biosynthesis, an overall decline would be expected coinciding with fruit AsA accumulation.

New milestone 1.21 – Complete characterisation of annual carbohydrate storage cycle in blackcurrant plants (31.03.07)

II. Determination of leaf export status

The capacity of leaves to export sugars will be determined during the period of AsA accumulation in fruit. Leaf discs will be lightly abraded and incubated with [U-^{14}C]sucrose, after incubation the discs will be extensively washed and exposed to X-ray film to determine the distribution of radiolabel. In developing leaves, a diffuse pattern of sucrose labelling would be expected as this leaf type is actively importing sucrose from plant reserves to allow leaf development. In fully mature exporting leaves, the radiolabel would be expected to accumulate in leaf veins which are actively concentrating sucrose for export to other parts of the plant (Fellows & Geiger, 1974).

New milestone 1.22 – Determine leaf export capacity during AsA accumulation in blackcurrant fruit (31.10.06)

This work will contribute to milestone 5.4 – Develop mechanistic understanding of AsA synthesis and distribution in *R. nigrum* which will remain unchanged with a completion date of 31.03.07.

2. Development of predictive markers for blackcurrant fruit AsA

Two complementary approaches will be adopted; a biochemical approach using both targeted and non-targeted measurements and a molecular approach in which the targets are defined as a result of work undertaken to understand the factors leading to accumulation of AsA in blackcurrant fruit.

I. Biochemical approach

A population of 200 seedlings consisting of a cross between Ben Gairn and breeding line S18-10-18 will be transferred to a controlled environment chamber and leaf tissue non-destructively sampled at different stages throughout development. Care will be taken to ensure that leaves are always sampled under the same conditions and at the same point in the diurnal cycle in order to minimise environmental variation. Several metabolites will be determined in leaf tissues.

a) Hydrophilic compounds will be extracted in perchloric acid using a method previously developed at SCRI for the quantification of a wide range of metabolites

from raspberry buds (DEFRA Project HH2203 NSF; http://www.defra.gov.uk/science/project_data/DocumentLibrary/HH2203NSF/HH2203NSF_3238_FRP.doc) and the following compounds quantified.

- AsA by HPLC – A simple correlation whereby a plant with high AsA in fruit at maturity has a generally high level of AsA in all tissues throughout development.
- Organic acids by HPLC – Levels of organic acids can provide information regarding respiratory activity, which produces AsA consuming reactive oxygen species. For example, genetically engineered tomato fruit with an alteration in expression of a respiratory enzyme had higher succinate and AsA (Nunes-Nesi *et al.*, 2005).
- Glutathione by HPLC – Glutathione is involved in the recycling of AsA and genetic engineering to specifically increase plant glutathione levels also had a positive impact on AsA (Foyer *et al.*, 1995).

New milestone 2.11a – Quantify AsA, organic acids and glutathione in leaf tissues of a 200 seedling population grown under a controlled environment (31.10.06)

b) Sequential aqueous and organic extraction will be undertaken on leaf tissues and several measurements of total antioxidant capacity (e.g., trolox equivalents antioxidant capacity, ferric reducing ability) will be undertaken. A number of antioxidants have been shown to protect AsA in vitro (Millar & Rice-Evans, 1997) and a higher general antioxidant capacity may result in higher levels of AsA in tissues.

New milestone 2.11b – Determine the antioxidant capacity of a 200 seedling population grown under a control environment (31.10.06)

c) Leaf tissue will be extracted in aqueous methanol using methodology developed at SCRI in order to allow the unbiased extraction of a large number of metabolites. Samples will then be analysed by both gas and liquid chromatography in conjunction with mass spectrometry to a profile of the metabolite content to be obtained consisting of in the region of 200-500 individual compounds. Statistical analyses will be conducted to determine whether any specific metabolites or groups of metabolites are suitable for the prediction of fruit AsA concentration.

New milestone 2.11c – Determine the metabolite profile of a 200 seedling population grown under a controlled environment (31.03.07)

d) Leaf tissue will be extracted in appropriate buffers and enzyme activities involved in AsA synthesis and recycling determined. If there are changes in gene sequences resulting in changes in enzyme regulation and kinetics these would be conserved between leaf and fruit tissue.

New milestone 2.11d – Determine critical enzyme activities involved in AsA synthesis and AsA turnover in leaf tissues of a 200 seedling population grown under controlled environment conditions (31.03.07)

II. Molecular approach

Work undertaken to date has clearly identified biosynthesis through the L-galactose pathway as a strong determinant of AsA concentration in ripe blackcurrant fruit. Likely rate limiting steps in AsA biosynthesis have been determined using a variety of methods. GDP-D-mannose pyrophosphorylase was identified as having a point mutation in *Arabidopsis thaliana* plants that were sensitive to ozone toxicity. The mutation resulted in a 35% reduction in enzyme activity but a 65% reduction in plant AsA concentration (Conklin *et al.*, 1999). Similarly, potato plants containing an antisense construct for the same enzyme had a much reduced AsA concentration (Keller *et al.*, 1999). GDP-D-mannose 3,5-epimerase activity was shown correlate with AsA concentration in a series of mutants of the colourless micro alga *Prototheca moriformis* (Running *et al.*, 2003) and enzyme activity (Wolucka *et al.*, 2001) and gene expression (Wolucka *et al.*, 2005) correlated with AsA biosynthesis in *A. thaliana*. Two other genes have very recently been identified as a result of gene mapping in mutant *A. thaliana* with low AsA phenotypes. These are GDP-L-galactose phosphorylase and L-galactose-1-phosphate phosphatase.

Initial work will examine the level of expression of the four genes in developing fruit of three different genotypes in order to determine whether differences in AsA biosynthetic capacity between genotypes is correlated with differences in gene expression. cDNA's for GDP-D-mannose pyrophosphorylase (truncated) and epimerase (full length) have already been isolated from blackcurrant fruit by the SCRI fruit genomics group, the other genes will be cloned using conserved regions identified from multiple sequence alignments. The

utility of such methods for the identification of rate limiting steps in antioxidant biosynthesis has been demonstrated in potato tubers at SCRI. Gene expression in potato cultivars with different carotenoid complements was examined (Morris *et al.*, 2004) which revealed targets for the subsequent metabolic engineering of potatoes with altered carotenoids profiles (Ducreux *et al.*, 2005).

New milestone 2.11e – Determine gene expression of four critical biosynthetic genes during fruit development in three blackcurrant genotypes (31.12.06)

If necessary, gene expression studies in further blackcurrant cultivars will be undertaken.

Secondary milestone 2.11 f – Determine gene expression of critical biosynthetic genes in a range of blackcurrant cultivars (31.06.07)

Genotype specific differences in AsA biosynthetic capacity are likely to arise as a result of either differences in gene expression or alternatively, differences is the regulatory or kinetic characteristics of the translated enzyme. The former may arise as a result in polymorphisms in the promoter region of the transcribed gene and the latter may arise as a result of polymorphisms in the coding region of the gene. High degrees of polymorphism have previously been identified in maize lines (Holland *et al.*, 2001) suggesting that promoter polymorphism might also be common in other species. In addition, previous work has shown that polymorphisms in the coding region of a tomato fruit specific invertase is correlated with Brix (Fridman *et al.*, 2004) demonstrating that mutations in enzyme encoding genes can have significant effects on fruit phenotypes. Furthermore, polymorphisms in the maize *Y1* gene encoding phytoene synthase could be associated with carotenoid content across 75 individual lines (Palaisa *et al.*, 2003). Whatever the mechanism, polymorphisms would be conserved amongst all tissues at all developmental stages and could therefore provide robust predictive markers for fruit AsA. In order to determine which genetic polymorphisms are associated with higher fruit AsA concentrations, the coding and promoter regions of a number of biosynthetic genes from high and low fruit AsA blackcurrant cultivars will be sequenced. It is envisaged that polymorphisms will be detected after sequencing very few cultivars however, in order to confidently associate particular polymorphisms with fruit AsA concentrations upwards of 10 cultivars of known fruit AsA concentration should be sequenced.

New milestone 2.11g – Identify polymorphisms in blackcurrant AsA biosynthetic genes associated with low or high fruit AsA (31.06.07)

Revised Milestones

A complete primary milestone list is supplied as Appendix 1

1.11 – Complete characterisation of superior lines from segregating population (DELETED)

1.16 – Complete biochemical and physiological characterisation of AsA accumulating mutants, if available (DELETED)

1.20 – Complete biochemical/physiological characterisation of new enhanced germplasm (DELETED)

1.21 – Complete characterisation of annual storage cycle in blackcurrant plants (31.03.07)

1.22 – Determine leaf export capacity during AsA accumulation in blackcurrant fruit (31.10.06)

2.11 – Identify robust correlative markers that can be used to predict berry AsA content in pre-fruiting stages (new completion date 31.06.07)

2.11a – Quantify AsA, organic acids and glutathione in leaf tissues of a 200 seedling population grown under a controlled environment (31.10.06)

2.11b – Determine the antioxidant capacity of a 200 seedling population grown under a control environment (31.10.06)

2.11c – Determine the metabolite profile of a 200 seedling population grown under a controlled environment (31.03.07)

2.11d – Determine critical enzyme activities involved in AsA synthesis and AsA turnover in leaf tissues of a 200 seedling population grown under controlled environment conditions (31.03.07)

2.11e – Determine gene expression of four critical biosynthetic genes during fruit development in three blackcurrant genotypes (31.12.06)

2.11f – Determine gene expression of critical biosynthetic genes in a range of blackcurrant cultivars (31.06.07; secondary milestone)

2.11g – Identify polymorphisms in blackcurrant AsA biosynthetic genes associated with low or high fruit AsA (31.06.07)

3.9 – Complete investigation on the effect of temperature on AsA production in blackcurrant bushes (new completion date 31.05.07)

5.3b – Complete grower field trial SCRI (DELETED)

5.4 – Develop mechanistic understanding of AsA synthesis and distribution in *R. nigrum* (new completion date 31.06.07)

5.5 – Deliver final report (30.09.07)

Deliverables

1. Molecular account for the phenotypic differences in fruit AsA levels in *R. nigrum*.
2. Biochemical and/or molecular markers for the early identification and selection of *R. nigrum* phenotypes with an acceptable fruit AsA concentration.
3. A comprehensive model to account for the mechanisms of AsA accumulation in blackcurrant fruit and inter-genotype differences.

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Revised milestone list

Task	Status	Objectives	Date	Comments
1.3	Original	Complete preliminary distribution and transport studies	31.10.03	Completed
1.7	Original	Complete distribution and transport studies	31.10.04	Completed
1.11	Original	Complete characterisation of superior lines from segregating population	31.03.05	Deleted
1.16	Original	Complete biochemical and physiological characterisation of AsA accumulating mutants (if available)	31.03.06	Deleted
1.20	Original	Complete characterisation of new enhanced germplasm	31.03.07	Deleted
1.21	New	Complete characterisation of annual storage cycle in blackcurrant plants	31.03.07	In progress
1.22	New	Determine leaf export capacity during AsA accumulation in blackcurrant fruit	31.10.06	In progress
2.5	Original	Identify correlative traits of berry AsA	31.10.04	Completed
2.8	Original	Finish screening mutagenised populations	11.02.05	Completed
2.9	Original	Identification of correlative traits	31.10.05	Completed
2.11	Original	Identification of AsA markers	31.10.06	In progress
2.11a	New	Quantify AsA, organic acids and glutathione in seedling leaf tissue	31.10.06	Part achieves 2.11
2.11b	New	Determine the antioxidant capacity of seedling leaf tissue	31.10.06	Part achieves 2.11
2.11c	New	Metabolic profiling seedling leaf tissue	31.03.07	Part achieves 2.11
2.11d	New	Determine AsA biosynthetic enzymes in seedling leaf tissue	31.03.07	Part achieves 2.11
2.11e	New	Determine AsA biosynthetic gene expression in three blackcurrant cultivars	31.12.06	Part achieves 2.11
2.11g	New	Identify polymorphisms in blackcurrant AsA biosynthetic genes	31.06.07	Part achieves 2.11
3.4	Original	Complete pilot experiment	31.03.03	Completed
3.7	Original	Identify suitable agronomic techniques	31.03.04	Completed
3.9	Original	Complete temperature studies	31.05.07	In progress, new completion date
4.3	Original	Complete data processing regional survey	31.03.04	Completed
4.5	Original	Complete analysis field trial	31.03.05	Completed
4.7	Original	Complete analysis of all field trials	03.04.06	In progress
5.3a	Original	Complete grower field trial (EMR)	07.12.06	In progress
5.3b	Original	Complete grower field trial (SCRI)	30.09.07	Deleted
5.4	Original	Develop mechanistic understanding	30.03.07	In progress, aided by 1.21, 1.22
5.5	Original	Deliver final report	30.09.07	No change