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Linking ascorbic acid production in *Ribes nigrum* with fruit development and changes in sources and sinks

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• *Background and Aims* Understanding the synthesis of ascorbic acid (L-AsA) in green tissues in model species has advanced considerably; here we focus on its production and accumulation in fruit. In particular, our aim is to understand the links between organs which may be sources of L-AsA (leaves) and those which accumulate it (fruits). The work presented here tests the idea that changes in leaf and fruit number influence the accumulation of L-AsA. The aim was to understand the importance of leaf tissue in the production of L-AsA and to determine how this might provide routes for the manipulation of fruit tissue L-AsA.
• *Methods* The experiments used *Ribes nigrum* (blackcurrant), predominantly in field experiments, where the source–sink relationship was manipulated to alter potential leaf L-AsA production and fruit growth and accumulation of L-AsA. These manipulations included reductions in reproductive capacity, by raceme removal, and the availability of assimilates by leaf removal and branch phloem girdling. Natural variation in fruit growth and fruit abscission is also described as this influences subsequent experimental design and the interpretation of L-AsA data.
• *Key Results* Results show that fruit L-AsA concentration is conserved but total yield of L-AsA per plant is dependent on a number of innate factors many of which relate to raceme attributes. Leaf removal and phloem girdling reduced fruit weight, and a combination of both reduced fruit yields further. It appears that around 50 % of assimilates utilized for fruit growth came from apical leaves, while between 20 and 30 % came from raceme leaves, with the remainder from 'storage'.
• *Conclusions* Despite being able to manipulate leaf area and therefore assimilate availability and stored carbohydrates, along with fruit yields, rarely were effects on fruit L-AsA concentration seen, indicating fruit L-AsA production in *Ribes* was not directly coupled to assimilate supply. There was no supporting evidence that L-AsA production occurred predominantly in green leaf tissue followed by its transfer to developing fruits. It is concluded that L-AsA production occurs predominantly in the fruit of *Ribes nigrum.*

Key words: L-Ascorbic acid, blackcurrant, fruit, *Ribes nigrum*, source sinks, vitamin C.

INTRODUCTION

Understanding the production of L-ascorbic acid (L-AsA) and its cellular roles has advanced considerably over the last decade (Wheeler *et al.,* 1998; Loewus, 1999; Agius *et al.*, 2003; Wolucka and van Montagu, 2003; Lorence *et al.*, 2004; Ishikawa *et al.*, 2006; Walker *et al.*, 2009) as has its accumulation in fruit (Hancock and Viola, 2005; Hancock *et al.*, 2007; Davies *et al.*, 2009; Cruz-Rus *et al.*, 2011). The importance of irradiance on fruit relative to leaves, in tomato, suggests that there is little linkage between leaves and fruits in the supply of L-AsA (Gautier *et al.*, 2009). While, for example, the manipulation of kiwi vine temperature supports fruit-based production, others have observed variability in long-distance phloem L-AsA transport from leaves to developing fruit (Giovanelli *et al.*, 1999; Franceschi and Tarlyn, 2002; Davey *et al.*, 2004; Richardson *et al.*, 2004). In apple, fruit L-AsA concentration was dependent on production which declines with maturation, despite L-AsA accumulating with increasing fruit weight (Li *et al.*, 2011). Tomato fruit show pectin polymer degradation as a source of precursors for L-AsA synthesis and accumulation via L-galactonic acid (Di Matteo *et al.*, 2010). What is clear are species differences in the mechanism by which total fruit L-AsA production is modulated during development; in some fruit, e.g. strawberry, melon and tomato, it remains constant while in others, e.g. apple and orange, it declines (see Imai *et al.*, 2009).

The aim of the work described here is to determine the role of green leafy tissues in the development and growth of fruits and how these processes influence L-AsA production and accumulation in fruit. We have used *Ribes nigrum* as a model plant because its fruit have high L-AsA concentrations and there is some knowledge of the pattern of biosynthesis and accumulation of L-AsA over time (Viola *et al.*, 2000; Walker *et al.*, 2009). What remains unclear is the location of fruit L-AsA synthesis and under what circumstances, if any, does fruit growth compete with L-AsA production.

We hypothesise that if leaf tissue is the primary source of either L-AsA or the precursory carbohydrates, then altering the source : sink ratio should influence fruit L-AsA concentration and its accumulation. Firstly, we present data which shows how L-AsA varies with plant developmental factors which

include the position on which fruit is borne, i.e. 'the age of the branch' and the influence of fruit number (within the raceme) on the inherent variability in fruit L-AsA concentration. These factors are known to influence fruit L-AsA accumulation in perennial crops (Ma and Cheng, 2003; Davies *et al.*, 2009). Variation in fruit L-AsA concentration due to fruit size is analysed, as this is known to show systematic variation. Experiments are also presented which are used to manipulate fruit yields, either during anthesis, or by adjusting sink size by removing developing fruits from within a raceme, to determine the effects on fruit L-AsA. A raceme is an inflorescence which is not branched being indeterminate, bearing pedicellate flowers on short pedicels (see ADAS, 1976). The raceme also develops leaves which arise from the flowering node and are considered to function as an autonomous unit, and a source of photoassimliates, particularly during early fruit development, within the raceme. These leaves are therefore a manipulatable source of both photoassimilates for fruit growth and possibly the precursors for leaf and/or fruit L-AsA production. This provides an experimental approach; by using a combination of phloem girdling and leaf removal, the sources of carbohydrates for fruit growth and L-AsA accumulation in *Ribes* can be determined.

MATERIALS AND METHODS

Plant material

Ribes nigrum 'Baldwin', 'Ben Lomond' and 'Ben Tirran' were obtained from commercial suppliers and 'Hedda' from Planteforsk, Kiseveien, Norway. The latter cultivar was used solely in a pot-based comparative experiment to understand differences of crop yield on innately low (60 mg L-AsA 100 g f. wt^{-1}) and high ('Baldwin' 220 mg L-AsA 100 g f. wt^{-1}) L-AsA-accumulating cultivars (Viola *et al.*, 2000). Potted plants were used in comparative experiments with 'Hedda', as this cultivar requires sufficient cold to achieve full reproductive development and this could only be guaranteed through artificial cold storage. All other plants were field grown using conventional crop spacing (3 m × 0·5 m) and subject to East Malling Research's standard pest and disease control programme for *Ribes nigrum*. Trickle irrigation and fertilizer were applied initially during establishment and then as required (see Davies *et al.*, 2009).

Effects of branch-bearing age on fruit yield and L-AsA concentration

Woody perennials show variation in fruit-setting potential and production associated with position ('age of wood') on which fruit is borne (see Robbie and Atkinson, 1994). Variation in fruit bearing was quantified with respect to fruit yield (fresh weight), fruit and L-AsA concentration and total L-AsA yield on a per plant basis. Ten similar plants of 'Baldwin' were selected and the number of racemes and fruit per raceme recorded in May, June and July. Fruit were harvested in late July from different ages of branch and size graded and frozen in liquid nitrogen prior to storage at −80 °C for L-AsA analysis.

Effects of natural variation in raceme fruit number and L-AsA concentration

Fruit number per raceme was recorded for eight 'Baldwin'plants selecting, at random, from each plant five large racemes (with between 8 and 14 berries) and five small racemes (with 2–5 berries). At harvest, fruit were separated based on size (<5·6 mm; 5·6–8 mm; 8–11·2 mm and >11·2 mm) and frozen in liquid nitrogen prior to storage at −80 °C for L-AsA analysis. Earlier measurements had shown that L-AsA concentration varied with fruit size (Davies *et al.*, 2009).

Effects of complete flower removal from entire plants on subsequent year's fruit L-AsA

Flowers were removed from 'Baldwin' plants and the production of fruit and L-AsA concentration determined the following cropping season. Plants which had all their flowers removed (prior to fertilization in early May) were compared with controls which had no flowers removed, using a ten-replicate completely randomized block design, with one plant per treatment per block. Fruit were harvested in late July recording total yield per plant and the fruit size distribution from sub-samples per plant. A sub-sample was also frozen in liquid nitrogen and stored at −80 °C for L-AsA analysis.

Effects of entire raceme removal, to different intensities per plant, on fruit yield and L-AsA concentration

To determine the effects of different numbers of racemes per plant, complete racemes were removed in varying degrees from 0 % to 80 %, over the entire plant. Flower removal (thinning) treatments were applied in mid-April, when the 'Baldwin' and 'Hedda' plants were at fruit development stage 'F3' (100 % flowers open) (see ADAS, 1976). The treatments were as follows: treatment 1, control (no thinning only natural abscission); 2, 20 % thinned; 3, 50 % thinned; 4, 80 % thinned. The potted plants were arranged on a free-draining gravel bed in a split-plot design: two cultivars × 4 thinning treatments × 6 replicates, with two main plots (cultivars) within each replicate block and four sub-plots (thinning treatments) within each main plot. Fruit samples were frozen in liquid nitrogen and stored at −80 °C for L-AsA analysis.

Effects of partial fruit removal within the raceme on fruit yield and L-AsA concentration

Ten field-grown 'Baldwin' plants had their potential crop yield adjusted by around 50 % by removing individual fruits from each raceme. Fruit were removed, in May during fruit set, from the distal end of each raceme, losing hierarchically the less-well developed fruits. The control treatment had no fruit removed. A completely randomized block design was used with each block separated by a guard plant. Fruit were harvested in late July and total yield per plant recorded. Sub-samples were used for fruit size grading and frozen in liquid nitrogen and stored at −80 °C prior to L-AsA analysis. Shoot extension growth was recorded during dormancy, along with new shoot growth and total shoot length per plant.

Effects of leaf removal on the subsequent year's fruit yield and L-AsA concentration

All leaves were removed from the experimental plants either at harvest (in July) or 1, 2 or 3 months later. The fifth treatment was the control plants, which were allowed to undergo natural winter defoliation. The experimental design was a completely randomized block with ten blocks. Analysis of fruit yield, fruit L-AsA concentration and total L-AsA per plant were determined the year following the defoliation. Fruit samples for different fruit sizes were frozen in liquid nitrogen and stored at −80 °C prior to L-AsA analysis.

Effects of raceme leaf removal and phloem girdling on fruit yield and L-AsA concentration

In late May, 30 'Ben Tirran' plants were selected and on each of three branches their 'raceme leaves' (leaves linked anatomically to the raceme) removed, leaving intact the terminal shoot leaves at branch apices. A further three branches, on the same plants, were selected for comparison and their raceme leaves left intact along with their terminal shoot leaves. Early observations suggested that this phase of fruit development was important in determining fruit size and final L-AsA concentration. Plants were paired and the branches on one of the pair subject to branch phloem girdling (xylem left intact). The effectiveness of the approach required practical skill to ensure that only phloem disruption occurred and not xylem cavitation. To ensure sufficient replication, more branches were girdled than needed for good statistical replication. Any branch which showed initial evidence of loss of leaf turgor was not used, as was the case for any later loss of branch viability. Only actively growing branches were used in the final analyses. The quantity of bark tissue to which the isolated raceme had access was cut to as consistent a stem length as possible to reduce supply variability**.** At both the upper and lower girdles, a complete ring of bark/phloem of around 3 mm was removed to ensure complete isolation of the raceme and its leaves. Fruit were harvested, from one of each of the branches with and without raceme leaves, for all the 30 plants in both late June and mid-July with the fruit being separated into different sizes and samples frozen in liquid nitrogen and stored at −80 °C for L-AsA analysis.

Fruit L-AsA analyses

Total fruit L-AsA concentration (L-dehydroascorbic acid plus L-ascorbic acid) was measured by HPLC-UV. Twenty grams frozen weight of berries were homogenized in a blender with [1 : 10] w/v volumes of 25 mM ammonium acetate buffer pH 4·0. The homogenate was ultracentrifuged (MSE Europa 24M) at 17 000 *g* at 4 °C for 15 min and supernatant collected. Three hundred microlitres of 5 % trifluoroacetic acid (Fluka UK) and 30 μL of 500 mM of the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (Fluka UK) were added to a 3-mL aliquot of supernatant and left at 4 °C for 30 min. Five hundred microlitres of this solution was pipetted into a Whatman Mini-UniPrep HPLC vial incorporating a 0·45-μm polypropylene filter. Ten microlitres of sample were injected into the HPLC.

HPLC conditions were as follows: Varian Chromsep Polaris C18-A 3 μ 150 × 2 mm column, fitted with a Chromsep 10 × 2·0 mm Polaris C18-A 3 μ guard cartridge; column temperature 30 °C; mobile phase 0·5 % trifluoroacetic acid 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 L-AsA was at 245 nm, and quantitative analysis was by an external standard calibration curve, generated from injecting varying concentrations of an L-ascorbic acid (Aldrich UK) standard solution. The external calibration methodology follows that of Walker *et al.* (2006).

Experimental design and statistical analysis

All experiments were designed in consultation with a qualified biometrician. Treatment differences were determined using ANOVA (Genstat software Ver. 9·1; Rothamsted Experimental Station, UK).

RESULTS

Effects of branch-bearing age on fruit yield and L-AsA concentration

As the age of branches which bore fruit increased for 'Baldwin', fruit yield significantly declined (Table 1); this was also true for the 'Ben Lomond' (data not shown). There were no significant differences in fruit L-AsA concentration with either cultivar, with total production of L-AsA per plant per bearing age declining significantly, but this was primarily determined by fruit yield not fruit L-AsA concentration (Table 1).

TABLE 1. *The effects of branch-bearing age for 'Baldwin' plants on fruit yield and L-AsA concentration and total L-AsA yield for different fruit sizes*

Fruit size (mm)	Branch fruit bearing age (years)	Fruit yield (g)	L-AsA concentration (mg g^{-1})	Total L-AsA (mg $plant^{-1}$)
8–11·2	1	39·8	1·81	74·2
	2	25·5	1·78	45·6
	3	6·0	1·70	10·1
	F. prob.	***	n.s.	**
	s.e.d. (d.f. = 7)	7·35	0·095	13·93
>11·2	1	26·2	1·60	42·1
	2	19·4	1·57	30·0
	3	6·6	1·79	10·4
	F. prob.	**	n.s.	**
	s.e.d. (d.f.= 7)	5·48	0·108	8·47
Total	1	66·0		116·3
	2	44·9		75·7
	3	12·6		20·5
	F. prob.	**		**
	s.e.d. (d.f. = 7)	11·83		20·82

F. prob., *F.* probabilities: **, 0·01; ***, 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

TABLE 2. *The effect of natural variation in raceme fruit number (large = 8–14, small = 2–5) for 'Baldwin' plants on L-AsA concentration and total L-AsA per fruit*

	L-AsA concentration (mg g^{-1} f. wt)		Total L-AsA per fruit (mg $fruit^{-1}$)	
Fruit size (mm)	Large raceme	Small raceme	Large raceme	Small raceme
>11·2	3·3	4·5	3·6	4·4
8–11·2	3·8	4·1	1·9	2·1
5·6–8	3·4	4·9	0·9	1·3
<5·6	5·8	6·7	0·7	0·6
F. prob.				
Raceme fruit number	**		**	
Fruit size	***		***	
Fruit number × size	n.s.		n.s.	
s.e.d. (d.f. = 47)				
Raceme fruit number	0·29		0·13	
Fruit size	0·40		0·18	
Fruit number × size	0·59		0·25	

F. prob., *F.* probabilities: **, 0·01;***, 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

Effects of natural variation in raceme fruit number and fruit L-AsA concentration

Despite significant differences in mean raceme fruit number (10, large racemes; 3·8, small racemes) the mean individual fruit sizes were similar at 493 and 468 mg f. wt, respectively, for large and small racemes (data not shown). L-AsA concentration in all fruit size categories was highest for the racemes with the smaller number of fruits (Table 2 for statistical analysis). Mean fruit L-AsA concentration for large and small racemes was 4·07 and 5·03 mg g^{-1} f. wt, respectively. L-AsA concentration based on fruit size alone was only significantly higher for the smallest-sized fruits in both raceme sizes (Table 2). Despite the higher L-AsA concentration in the small fruit, total L-AsA per fruit was greater in larger fruits. Mean total L-AsA per fruit was 1·74 mg and 2·11 mg for the large and small racemes, respectively. Total L-AsA for the large and small racemes was 19 and 7 mg, respectively.

Effects of complete flower removal from entire plants on the subsequent year's fruit L-AsA

The influence of flower removal on shoot growth was measured at the end of the growing season in which the flowers had been removed. Despite no significant differences in the number of newly initiated shoots, mean shoot length was significantly greater for plants which had been deflowered at anthesis, but this had no effect on total shoot growth per plant (Table 3). Fruit production, in the following year for plants which had been deflowered, was significantly greater in all fruit sizes categories, except the largest (Table 4). No deflowering treatment influenced fruit L-AsA concentration, but total amount of L-AsA per plant was significantly greater for the previously deflowered plants (Table 4).

TABLE 3. *The effects of complete flower removal, at anthesis, the previous year for 'Baldwin' plants, on number of new shoots, mean shoot length and total shoot length per plant*

	No. of new shoots	Mean shoot length (cm)	Total shoot length per plant (cm)
Control	54·1	22·8	1245
Removed	64·4	25·9	1675
F. prob.	n.s.	*	n.s.
s.e.d. (d.f. = 39)	10·53	1·03	250·2

F. prob., *F.* probabilities: *, 0·05; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

Effects of entire raceme removal, to different intensities per plant, on fruit yield and L-AsA concentration

When entire racemes were removed at varying intensities within the plant (0–80 %) from a low L-AsA-accumulating cultivar, 'Hedda' and a high L-AsA-accumulating cultivar, 'Baldwin', there were, as expected, significant reductions in fruit yield (Table 5). Comparing actual yields with predicted yields, based on a relative multiplication factor derived from the percentage of racemes removed by the different treatment intensities (i.e. normalized yields), 'Hedda', had actual yields similar to the expected, irrespective of the level of raceme removal. This was not the case with 'Baldwin' where yields were greater than expected in regard to the level of raceme removal. Despite changes in fruit yields per plant and differences in 'compensatory' fruit growth between the two cultivars, there were no significant treatment effects on fruit L-AsA concentration, only cultivar differences (Table 5).

Effects of partial fruit removal within the raceme on fruit yield and L-AsA concentration

Flowers thinned by 50 % for 'Ben Lomond' showed a mean yield reduction of slightly <50 %, while for 'Baldwin' there was only a reduction in mean total yield with raceme thinning on fruit within the smaller size category (Tables 6 and 7). Despite inducing a reduction in fruit yield by raceme thinning, there were only small differences in 'Baldwin' fruit L-AsA concentration and only for the smaller fruit category. Within the larger fruit size category, yields increased significantly, but again fruit L-AsA concentrations were only elevated, as with 'Ben Lomond', within the smaller fruit size category (Tables 6 and 7). For both cultivars, differences in total L-AsA per plant were due prominently to differences in fruit yield, but the total compensatory effect on fruit yield and total amount of L-AsA was much greater with 'Baldwin' (Tables 6 and 7).

Removal of fruits rather than flowers during development, by around 50 %, reduced fruit yields per plant significantly only within the smaller-sized fruit (Table 8). There was no significant thinning effect on total fruit yield per plant, nor was there any thinning effect on fruit L-AsA concentration; however, an increase in fruit yield significantly increased

TABLE 4. *The effects of complete flower removal for 'Baldwin' plants, the previous year, on fruit yield in different fruit sizes (mm), total fruit yield, L-AsA concentration and total L-AsA yield per plant*

Treatment	Fruit yield (g)				L-AsA concentration (mg g^{-1} f. wt)			Total L-AsA per plant (mg $plant^{-1}$)
	5·6–8	8–11·2	>11·2	Total	5·6–8	8–11·2	>11·2	
Control	122	598	578	1298	2·99	3·19	3·15	4077
Removed	245	961	859	2065	2·93	3·12	3·11	6306
F. prob.	*	*	n.s.	*	n.s.	n.s.	n.s.	*
s.e.d. (d.f. = 9)	43·5	129·5	130·1	290·5	0·011	0·075	0·076	949·4

F. prob., *F.* probabilities: *, 0·05; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

TABLE 5. *The effects of entire raceme removal, at anthesis†, for 'Hedda' and 'Baldwin' plants, on total crop yield (g), yield normalized to the none treatment, and L-AsA concentration for berries of size 8–11 mm and >11·2 mm*

Raceme removal (% total raceme number)	Fruit yield (g)		Normalized yield (%)		L-AsA concentration (mg g^{-1}) 8–11·2 mm		L-AsA concentration (mg g^{-1}) >11·2 mm	
	Hedda	Baldwin	Hedda	Baldwin	Hedda	Baldwin	Hedda	Baldwin
None	204	152	100	100	0·98	1·93	0·83	1·83
20 %	146	139	72	91	1·40	1·95	0·73	1·87
50 %	93	85	46	56	1·17	1·67	0·79	1·72
80 %	56	70	27	46	1·22	2·08	0·76	1·76
	F. prob.	s.e.d.			*F.* prob.	s.e.d.	*F.* prob.	s.e.d.
Cultivar (d.f. = 5)	n.s.	17·4			***	0·104	***	0·052
Thinning (d.f. = 30)	***	24·0			n.s.	0·198	n.s.	0·052
Cult × thin (d.f. = 30)	n.s.	33·9			n.s.	0·281	n.s.	0·082

F. prob., *F.* probabilities: ***, 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.
†Raceme removal was carried out when the flowers were fully open.

TABLE 6. *The influence of partial raceme removal, at anthesis, for 'Ben Lomond' plants, on fruit yield, fruit L-AsA concentration and total L-AsA yield, after separation into different fruit sizes*

Fruit size (mm)	Treatment	Fruit yield (g)	L-AsA conc. (mg g^{-1})	Total L-AsA (mg $plant^{-1}$)
8–11·2	No thinning	72	2·10	152
	50 % thinning	26	1·87	50
	F. prob.	***	**	***
	s.e.d. (d.f. = 11)	10·1	0·059	22·5
>11·2	No thinning	263	2·00	524
	50 % thinning	159	1·89	302
	F. prob.	*	n.s.	**
	s.e.d. (d.f. = 11)	35·0	0·072	69·6
Total	No thinning	335	0·676	676
	50 % thinning	186	0·353	353
	F. prob.	**	**	**
	s.e.d. (d.f. = 11)	40·7	0·0847	85·1

F. prob., *F.* probabilities: *, 0·05; **, 0·01; ***, 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

total L-AsA production per plant. Fruit removal had little effect on subsequent shoot growth, with only a small difference in mean shoot length measured (Table 9).

Effects of leaf removal on the subsequent year's fruit yield and L-AsA concentration

Leaf removal showed that, in general, at different times prior to natural defoliation, the earlier leaves were removed the greater the reduction in fruit yield, irrespective of fruit size category (Table 10). The only significant differences in fruit L-AsA were for the smallest-sized fruit (5·6–8 mm). Total plant L-AsA was therefore a reflection of differences in total fruit yield not L-AsA concentration (Table 10).

Effects of raceme leaf removal and phloem girdling on fruit yield and L-AsA concentration

Analyses of fruit growth of 'Ben Tirran' in mid-June showed that in the interim 22 d, since applying the treatments, despite fruit fresh weight increasing, raceme leaf removal severely reduced mean fruit weight and fruit number per branch. Phloem girdling on branches where raceme leaves were retained, showed mean fruit yields of 84 and 65 g f. wt (total fruit numbers of 301 and 274), for the non-girdled and girdled branches, respectively (Table 11). This is equivalent to a 23 % and a 9 % reduction in fruit weight and fruit number, respectively. Branches where raceme leaves were removed had mean fruit yields of 40 and 25 g f. wt (total fruit numbers of 194 and 123) for the non-girdled and

TABLE 7. *The influence of fruit thinning*[†]*, within a raceme, for 'Baldwin' plants, on fruit yield, fruit* L-*AsA concentration and total L-ASA yield, after separation into different fruit sizes*

Fruit size (mm)	Treatment	Fruit yield (g)	% fruit yield (angular transformed)	L-AsA conc. (mg g^{-1})	Total L-AsA (mg $plant^{-1}$)
8–11·2	No thinning	890	60·8	2·78	2481
	50 % thinning	651	48·0	2·64	1710
	F. prob.	n.s.	*	*	*
	s.e.d. (d.f. = 11)	121·1	3·54	0·059	330·8
>11·2	No thinning	298	29·2	2·40	794
	50 % thinning	530	42·0	2·43	1272
	F. prob.	n.s.	*	n.s.	n.s.
	s.e.d. (d.f. = 11)	106·6	3·54	0·057	265·3
Total	No thinning	1188			3233
	50 % thinning	1181			2982
	F. prob.	n.s.			n.s.
	s.e.d. (d.f. = 11)	177·0			443·2

F. prob., *F.* probabilities: *, 0·05; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.
[†]Fruit were removed after fruit set and from the lower part of the raceme.

TABLE 8. *The effects removing 50% of individual fruit within all racemes, for 'Baldwin' plants, on the fruit yield of berries of different sizes (mm), total yield,* L-*AsA concentration and total* L-*AsA yield per plant*

Treatment	Fruit yield (g)				L-AsA concentration (mg g^{-1})			Total L-AsA (mg $plant^{-1}$)
	5·6–8	8–11·2	>11·2	Total	5·6–8	8–11·2	>11·2	
Control	20	537	316	874	2·57	3·36	2·66	2660
50 % removal	10	312	356	679	2·90	3·11	2·54	1930
F. prob.	**	***	n.s.	n.s.	n.s.	n.s.	n.s.	*
s.e.d. (d.f. = 11)	3·0	51·6	66·5	102·4	0·149	0·132	0·129	327·0

F. prob., *F.* probabilities: *, 0·05; **, 0·01; ***, 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

TABLE 9. *The effects of removing 50% of fruit within all racemes, for 'Baldwin' plants, on the number of new shoots, mean shoot length and total shoot length per plant*

Treatment	No. of new shoots	Mean shoot length (dm)	Total shoot length (cm)
Control (unthinned)	24	3·44	828
Thinned to 50 %	23	3·85	872
F. prob.	n.s.	*	n.s.
s.e.d. (d.f. = 11)	2·0	0·16	82

F. prob., *F.* probabilities: *, 0·05; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

girdled branches, respectively (Table 11). This is equivalent to a 37 % reduction in both fruit weight and number. Measurements of fruit L-AsA showed little evidence that any of the four treatments influenced fruit L-AsA concentrations (Table 12). Differences in the yield of L-AsA per branch were apparent, and these were again due primarily to differences in fruit yield.

DISCUSSION

An explanation of how total fruit L-AsA is modulated during fruit development may differ with species (Imai *et al.*, 2009). L-AsA is detected in leaf phloem, but what remains unclear is what contribution long-distance transport from potential sources, such as leaves, makes to the pattern and amount of AsA that accumulates in fruit tissues at maturity (Franceschi and Tarlyn, 2002). Despite contrary evidence, albeit not universal, it is important to understand implications of altering the fruit to leaf area ratio should it impact on fruit L-AsA accumulation at harvest. However, before we can develop our understanding of these processes there is a need to clarify the innate variability which occurs with fruit development, particularly as this influences fruit L-AsA concentration, which is hierarchically apparent at different levels with many fruit, and particularly so for perennial woody crops (Hancock *et al.*, 2007; Davies *et al.*, 2009).

Reproductive performance with respect to the initiation and development of floral buds is critical in maximizing yields per plant, but it is known to vary particularly in perennial crops in a systematic way. Predictable variation in reproductive performance in relation to factors such as 'anatomical age' or 'positional' effects are generally well described for crops

TABLE 10. *The effects of total leaf removal on 'Baldwin' plants, at different times relative to natural defoliation, on fruit yield of berries of different sizes (mm), total fruit yield, L-AsA concentration per fruit and total L-AsA yield per plant*

	Fruit yield (g)				L-AsA concentration (mg g^{-1})			
Treatment	5·6–8	8–11·2	>11·2	Total	5·6–8	8–11·2	>11·2	Total L-AsA (mg $plant^{-1}$)
At harvest	82	352	174	608	3·62	3·65	3·37	2154
One month later	84	436	279	798	3·56	3·54	3·56	2818
Two months later	123	583	386	1092	3·21	3·41	3·16	3585
Three months later	121	452	245	818	3·23	3·40	3·35	2734
Control	139	665	405	1210	3·16	3·41	3·13	3965
F. prob.	*	**	*	**	***	n.s.	n.s.	*
s.e.d. (d.f. = 34)	20·1	87·8	80·3	166·0	0·110	0·141	0·178	541·9

F. prob., *F.* probabilities: * 0·05, ** 0·01 and *** .001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

within the *Malus*, *Prunus* and *Pyrus* genera (see, for example, Ferree and Warrington, 2003). This is not true, however, for *Ribes* where yield enhancement relies on generally unsupported anecdotal evidence. Our data show that firstly there is a considerable influence of anatomical age (branch age of flower bearing) on fruit yield with a highly significant decline, at least for 'Baldwin', of around 78 % on comparing racemes on 1-year-old with 3-year-old branches. Fruit yield from 1-year-old racemes was about 53 % of the total yield per plant, while that from 3-year-old racemes was 11 %. Despite this decline in yield having a significant impact on the total amount of L-AsA per plant (fruit L-AsA concentration multiplied by the total raceme fruit weight), differences in total L-AsA were solely due to anatomical age differences in fruit yield and not in fruit L-AsA concentration, irrespective of fruit size within the raceme.

Fruit number per raceme varies naturally and is dependent on the initial number of flower primordia present in developing buds and subsequently on their ability to be fertilized. Failure for all flowers (ovules) within a raceme to be fertilized and set fruit is not uncommon and fruitlets can abscise post-apparent fertilization (Denisow, 2003). This is typically evident with the loss of the more apical fruitlets within the raceme, these being the least well developed within the raceme. Such natural variation in fruit raceme number has the potential to alter the plant's sink capacity by reducing the assimilate demands from both fruit growth and perhaps fruit L-AsA production. Analysis of naturally large racemes (containing 8–14 individual fruits) and comparing these with small racemes (2–5 individual fruits) showed that the mean fruit sizes between these different racemes were similar (approx. 480 mg). Fruit L-AsA concentration was higher in all fruit sizes for those on the racemes with fewer fruits, but total L-AsA was greater for the racemes with the larger number of fruits (19 mg compared with 7 mg of L-AsA). The implications are that it is fruit number that determines total L-AsA yield per plant.

To test the hypothesis that L-AsA is exported from leaves and subsequently accumulates in fruit, the leaf area to fruit ratio was manipulated to alter the supply of photoassimilates and L-AsA precursors to developing fruit. Available leaf area (source) and fruit number (sink) was changed through a series of experiments where entire bushes had their reproductive capacity terminated for a season to determine the impact on fruit yields and L-AsA the following year. It is assumed that assimilates in the absence of fruit would be diverted to shoot growth or 'storage' as they are with other perennial plants. Whole racemes, during anthesis and later when fruit were developing, after fertilization, have also been removed at various intensities to create a varied fruit sink.

When fruit number was adjusted early in the development cycle (at anthesis) there was, as expected, a proportional reduction in fruit yield in direct response to the level of raceme removal, irrespective of cultivar. There was evidence that 'Baldwin' compensated to a greater extent, with respect to fruit growth and import of assimilates, apparent as an increase in the size of the remaining fruit, this was not the case with the 'Hedda' (see Toldam-Andersen and Hansen, 1993). Despite a reduction in fruit number per plant there was no change in fruit L-AsA concentration irrespective of cultivar. It is at this stage of fruit development that the accumulation of L-AsA is at its greatest (Viola *et al.*, 2000). When a reduction in fruit number was imposed later in the fruit development cycle, i.e. the removal of complete racemes at a level of 50 % per plant, the potential reduction in yield was much <50 % (only 22 % reduction). This compensation was due to an increase in the size of the remaining fruits but in the absence of any change in fruit L-AsA concentration. Experiments with 'Ben Lomond' and 'Baldwin', where 50 % of the fruit within a raceme was removed earlier in the fruit development cycle, were hypothesized as likely to show greater potential for compensatory growth. We suggest that fruit removal during earlier development is more likely to impact on L-AsA accumulation (see Viola *et al.*, 2000); particularly if the fruit are not the primary source of L-AsA and fruit sink competition for L-AsA exists. Again, 'Baldwin' showed the greater ability to compensate for a reduction in fruit raceme number by increasing fruit size, particularly with respect to the largest-size berries within the raceme. There was, however, no significant change to the fruit L-AsA concentration.

A change in the supply or availability of assimilates, for fruit growth and L-AsA production was investigated through the manipulation of raceme leaf area and phloem girdling. It was assumed that raceme leaves, due to their earlier appearance in the fruit development cycle in the absence of apical leaves, provide assimilates to sustain fruit set and earlier growth. Evidence shows *Ribes* L-AsA accumulation leads to

TABLE 11. *Mean total fruit fresh weights and fruit number for different fruit sizes per branch for 'Ben Tirran' subject to raceme leaf removal (leaves present or leaves removed) and basal branch girdling*

	Fruit (g f. wt) per branch								Mean fruit no. per branch							
	<5·6 mm		5·6–8 mm		>8 mm		Total		<5·6 mm		5·6–8 mm		>8 mm		Total	
	NG	G	NG	G	NG	G	NG	G	NG	G	NG	G	NG	G	NG	G
Leaves present	3·6	3·3	34	37	46	24	84	65	22	41	152	174	126	59	301	274
Leaves removed	3·0	2·8	27	18	11	4	40	25	35	37	127	76	32	11	194	123
	F. prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.
Girdling (d.f. = 34)	n.s.	0·9	n.s.	4·1	**	4·5	*	7·7	*	4·6	n.s.	18·3	***	12·2	n.s.	27·8
Leaf (d.f. = 34)	n.s.	0·9	**	4·1	***	4·5	***	7·7	n.s.	4·6	**	18·7	***	12·2	***	27·8
Gird. × leaf (d.f. = 28–34)	n.s.	1·3	n.s.	5·8	n.s.	6·4	n.s.	10·9	n.s.	6·5	n.s.	26·9	n.s.	17·2	n.s.	39·2

NG, Not girdled; G, girdled.
F. prob., *F.* probabilities: *, 0·05; ** 0·01; ***, 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

TABLE 12. *Mean fruit L-AsA concentration, mean L-AsA content per fruit for different fruit sizes and the total L-AsA yield per branch for 'Ben Tirran' plants subject to a combination of raceme leaf removal (leaves present or leaves removed) and basal branch girdling*

	Mean L-AsA concentration (mg g f. wt^{-1})						Total L-AsA yield per branch (mg)		Mean L-AsA amount per fruit (mg $fruit^{-1}$)					
	<5·6 mm		5·6–8 mm		>8 mm				<5·6 mm		5·6–8 mm		>8 mm	
	NG	G	NG	G	NG	G	NG	G	NG	G	NG	G	NG	G
Leaves present	4·8	5·1	5·0	5·6	4·6	5·8	383	360	0·7	0·4	1·1	1·2	1·6	2·3
Leaves removed	4·7	4·7	4·9	4·8	5·3	4·8	197	115	0·4	0·4	1·0	1·2	1·7	1·7
	F. prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	*F.* prob.	s.e.d.	F.prob.	s.e.d.
Girdling (d.f. = 34)	n.s.	0·23	n.s.	0·24	n.s.	0·33	n.s.	37·2	n.s.	0·16	n.s.	0·10	n.s.	0·16
Leaf (d.f. = 34)	n.s.	0·23	n.s.	0·24	n.s.	0·33	***	37·2	n.s.	0·16	n.s.	0·10	n.s.	0·16
Gird. x leaf (d.f. = 28–34)	n.s.	0·32	n.s.	0·34	*	0·48	n.s.	52·7	n.s.	0·23	n.s.	0·15	*	0·23

NG, Not girdled; G, girdled.
F. prob., *F.* probabilities: * 0·05, ** 0·01 and *** 0·001; n.s., non-significant.
s.e.d., Standard error of differences between the means; d.f., residual degrees of freedom for comparison.

initially high fruit L-AsA concentrations which are subsequent diluted by fruit expansion (Viola *et al.*, 2000). It was also assumed that phloem girdling by 'bark ringing' would terminate any phloem-based transport of assimilates from green source tissues, such as leaves. This assumption is based on work with other crops (Hancock *et al.*, 2007), including potato (Tedone *et al.*, 2004) and arabidopsis and *Medicago sativa* (Franceschi and Tarlyn, 2002). Girdling was applied to minimize the amount of tissue and the supply of stored carbohydrates from phloem/bark for fruit growth. Raceme leaf removal was shown to be closely linked to a reduction in fruit fresh weight and fruit number. Phloem girdling of branches also reduced fruit size. While a combination of raceme leaf removal and phloem girdling reduced fruit size further. These results show that around 50 % of the assimilates utilized for fruit growth came from the leaves present on apical shoots (non-raceme leaves), while between 20 and 30 % came from raceme leaves, with the remainder most likely to come from 'storage' (woody tissues and phloem within the branch girdle region). It is, however, assumed that severing of the phloem did not directly induce metabolic or transport changes which reduced fruit growth independently of assimilate supply. Importantly, despite an ability to manipulate the availability of assimilates and stored carbohydrates, variation in either source had no effect on fruit L-AsA concentration. Phloem severing, on girdling, equally did not elevate fruit L-AsA concentration as might be expected if the fruit were subject to other forms of stress (Smirnoff, 1996; Conklin, 2001). Hancock *et al.*, (2007) also suggest that the strict control of L-AsA entry into the phloem, in *Ribes*, rules it out as a significant source of fruit L-AsA. They have also suggested that leaf capacity for L-AsA synthesis is in decline when sink (fruit) accumulation is at its highest. We conclude that the notion L-AsA biosynthesis was directly, or solely, dependent on raceme leaf assimilates or stored stem carbohydrates is not supported. These results further support the notion that fruit L-AsA biosynthesis in *Ribes* is not directly coupled to carbohydrate substrate supply (Hancock *et al.*, 2007). Also, there does not appear to be a measurable level of precursor (assimilates or stored carbohydrates) competition which significantly influences L-AsA distribution between fruits within the raceme.

The predominance of genetic control of L-AsA concentration in fruit through a perhaps (Walker *et al.*, 2009), as yet, not fully defined regulatory network, with L-AsA recycling monodehydroascorbate and dehydroascorbate reductases, provides the capacity to maintain cell redox and cope with oxidative (environmental) stress (Davey *et al.*, 2006; Curz-Rus *et al.*, 2011). Equally, there was no evidence that L-AsA production occurs predominantly in green leaf tissue prior to direct transfer of L-AsA to accumulating fruits (Walker *et al.*, 2009).

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