Genotypic Influence on L-Ascorbic Acid Accumulation in Blackcurrant (*Ribes nigrum*) Fruit

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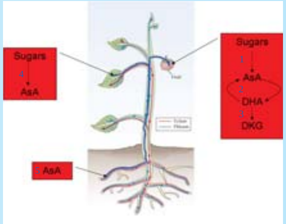
Blackcurrants are widely grown throughout Europe where they are the most commercially important bush fruit with annual production in excess of 550,000 metric tonnes[1]. The vast of majority of the blackcurrant crop is used for processing hence specific fruit quality components are becoming increasingly sought after, in particular the requirement for a high L-ascorbic acid (AsA) content[2]. In the present work, we address the question of what mechanisms are responsible for AsA accumulation in blackcurrant fruit (Fig. 1) of low, medium and high AsA genotypes. We conclude that the most likely source of phenotypic difference is the capacity for AsA synthesis from sugars imported into the fruit.

Figure 1
Potential mechanisms affecting AsA concentration of blackcurrant fruit



A number of mechanisms could affect the AsA concentration of sink tissues such as fruit. These include; 1. *In situ* biosynthesis from imported carbon 2. Recycling of oxidised AsA 3. Hydrolysis of DHA and loss from the fruit AsA pool 4. Synthesis in source leaves and transport to fruit via phloem 5. Mobilisation of stored AsA DHA = dehydroascorbic acid, DKG = 2,3-Diketogulonic acid

Materials and Methods

Plant Material and Growth Conditions

Ribes nigrum cultivars Hedda and Baldwin and genotype 8982-6 were grown in the field at Invergowrie, Dundee and subjected to standard commercial fertiliser and pesticide regimes. In the three years 2002/04 inclusive, ripe fruit AsA content was 71 ± 21, 196 ± 9 and 258 ± 25 mg gFW^{-1} for Hedda, Baldwin and 8982-6, respectively

Extraction and Measurement of AsA

AsA was extracted from fresh or lyophilised tissues in 5% metaphosphoric acid containing 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). After centrifugation, total AsA was quantified in the supernatant by HPLC with diode array detection at 245 nm[3]. Radioactive AsA was extracted in 5% perchloric acid containing 5 mM TCEP. [^{14}C]AsA was partially purified on SAX cartridges prior to quantification by HPLC with radioflow detection[4]. Phloem AsA was collected using the EDTA exudation technique[5] with minor modifications (15 mM EDTA, 1 mM TCEP, pH 6.5). In control exudates, EDTA was replaced with 5 mM $CaCl_2$.

Results

Figure 2
Changes in tissue AsA throughout growth cycle

- Phenotypic differences primarily confined to fruit
- Insufficient AsA storage to account for annual fruit accumulation

Whole plants were harvested at the times indicated, divided into the appropriate tissues and AsA extracted and quantified by HPLC. Data was normalised to plants comprising a total of 200 g dry weight at fruit harvest ± SE, n = 3.

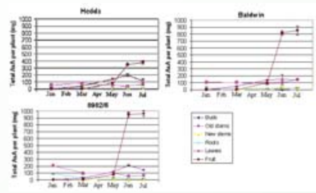


Figure 3
HPLC chromatogram of blackcurrant leaf exudates

- In common with other plants[4], blackcurrant phloem contains AsA
- Potential source of fruit AsA
- Technically difficult to quantify transport contribution

Leaf exudates were collected for 90 min. At the end of incubation, AsA was stabilised by addition of an equal volume of MPA/TCEP prior to HPLC analysis[3]. Traces shown are absorbance at 245 nm of —, EDTA exudation solution; —, $CaCl_2$ exudation solution; —, standard AsA. Inserts show absorption spectra of the peaks indicated.

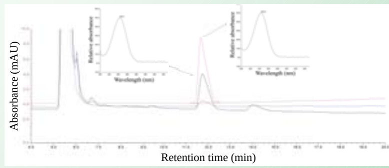


Table 1
Effect of potential precursors on fruit AsA content

- Only precursors of the L-Gal pathway[7] enhance fruit AsA content
- No evidence for operation of salvage (D-GalUA) pathway in immature fruit

In previous work, we demonstrated that blackcurrant fruit accumulate AsA at the early stages of development[6] so small green fruit were analysed for their potential to synthesise AsA from precursors of several different pathways (Fig. 4). Fruit were bisected and incubated for 24 h in a solution of 50 mM MES pH 6.5, 300 mM mannitol supplemented with 25 mM of the appropriate precursor. Fruit were extracted and AsA content estimated. Data show mean ± SE, n = 3. * P < 0.05, ** P < 0.01.

	AsA Concentration (mg gFW^{-1})		
Substrate	Hedda	Baldwin	8982-6
No additions	1.37 ± 0.04	3.59 ± 0.21	3.39 ± 0.24
D-glucose	1.51 ± 0.09	3.85 ± 0.24	3.94 ± 0.37
L-galactose	3.31 ± 0.37*	6.88 ± 0.12**	5.13 ± 0.49
L-galactonolactone	2.14 ± 0.09**	4.48 ± 0.07*	4.74 ± 0.27*
L-gulose	1.40 ± 0.07	3.96 ± 0.20	3.79 ± 0.22
L-gulonolactone	1.53 ± 0.09	3.93 ± 0.28	3.65 ± 0.26
myo-inositol	1.45 ± 0.14	3.78 ± 0.10	3.77 ± 0.18
D-galacturonic acid	1.46 ± 0.11	3.68 ± 0.03	3.77 ± 0.08

Figure 4
Schematic of proposed AsA biosynthetic pathways

Four potential routes for AsA biosynthesis have been proposed[7]. The L-Gal pathway is a *de novo* route for which much evidence has accrued since its discovery in 1998. The L-Gul pathway was proposed following *in vitro* analysis of GDP-D-Man-3,5-epimerase kinetics which showed that both GDP-L-Gal and GDP-L-Gul were reaction products. The MI pathway was proposed following the observation that overexpression of *myo*-inositol oxygenase results in enhanced AsA content in *Arabidopsis*. Evidence from strawberry suggests the D-GalUA pathway is a salvage pathway operating primarily in fruit.

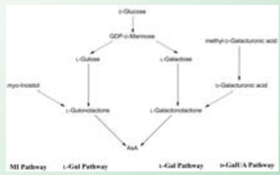
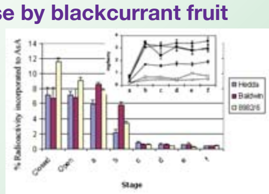


Figure 5
Biosynthesis of [^{14}C]AsA from D-[U-^{14}C]mannose by blackcurrant fruit

- Decline in AsA biosynthesis throughout fruit maturation
- Correlation between biosynthesis and accumulation during fruit maturation
- Correlation between fruit AsA content and biosynthetic capacity in individual genotypes



Blackcurrant fruit were harvested at six different stages of development6 and incubated with the L-Gal pathway precursor D-[U-^{14}C]mannose as previously described[4] with the exception that the buffer was as described for table 1. [^{14}C]AsA was extracted and estimated as previously described[4]. Data are mean activity recovered in [^{14}C]AsA ± SE, n = 3. Inset shows AsA accumulation in fruit of six blackcurrant cultivars[6].

Table 2
Turnover of fruit AsA pools

- Low AsA turnover (cf. 13% h^{-1} in pea embryonic axes[8])

Fruit were incubated in MES/mannitol (table 1) supplemented with 150 μM L-[1-^{14}C]AsA (S.A. 13 mCi $mmol^{-1}$) for 2 h. At the end of the incubation period, tissue was thoroughly washed in fresh MES/mannitol and either extracted immediately or incubation continued for a further 24 h prior to extraction. AsA turnover was calculated from changes in the specific activity of the fruit AsA pool after removal of label. Data are expressed as mean turnover h^{-1} ± SE, n = 3.

Genotype	% AsA pool turned over h^{-1}
Hedda	1.61 ± 0.07
Baldwin	2.68 ± 0.19
8982-6	0.73 ± 0.10

Conclusions

- Fruit AsA must be synthesised each year
- Potential contribution from AsA synthesised in leaves and imported via the phloem
- Low rates of fruit AsA turnover
- Correlation between fruit biosynthetic capacity and AsA contents of individual genotypes

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