

## Effects of Cultivar, Fruit Number and Reflected Photosynthetically Active Radiation on *Fragaria* × *ananassa* Productivity and Fruit Ellagic Acid and Ascorbic Acid Concentrations

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- **Background and Aims** A number of strawberry varieties were surveyed for their total ellagic acid concentration, and attempts were made to determine if ellagic acid and ascorbic acid concentrations of two strawberry cultivars could be increased by polythene reflective mulches.
- **Methods** After adjusting crop yields and cultivation using polythene mulches with two different PAR reflective capacities, field- and polytunnel-grown strawberries were analysed for ellagic acid and ascorbic acid concentrations by HPLC. Comparative measurements of yield and fruit quality were determined along with plant developmental changes.
- **Key Results** Ellagic acid concentration varied widely with strawberry cultivar (60–341 µg g<sup>-1</sup> frozen weight), as did the ratio of conjugated ellagic acid : free ellagic acid. Also, there was significant year-to-year variation in total ellagic acid concentration with some cultivars. Mulches with different reflective capacities impacted on strawberry production; highly reflective mulches significantly increased growth and yield, the latter due to increases in fruit size and number.
- **Conclusions** Highly reflective mulches significantly increased total concentrations of ellagic acid and ascorbic acid relative to control in fruit of different cultivars. The potential of agronomic practices to enhance the concentration and amounts of these important dietary bioactive compounds is discussed.

**Key words:** Ellagic acid, ellagitannins, ascorbic acid, *Fragaria*, PAR, everbearers, June bearers, light reflectance, reflective mulch, strawberries, vitamin C.

### INTRODUCTION

Strawberry production is a major part of the European and UK soft fruit industry with a home production value of £96m in 2003 (Defra, 2003). Research programmes aimed at improving the quality of strawberry fruit are important. Consistent and clear epidemiological evidence indicates the importance of increased consumption of fruit and vegetables in improving human health (Block *et al.*, 1992; Steinmetz and Potter, 1996; Kris-Etherton *et al.*, 2002). We are now beginning to attach increased importance to aspects of fruit quality related to the health benefits of fruit such as strawberry. These fruits contain high concentrations of potentially beneficial phytochemicals and antioxidants, such as ascorbic acid (vitamin C) and ellagic acid (Potter, 1997; Eastwood, 1999; Urquiaga and Leighton, 2000). Ascorbic acid, present in high concentrations, provides an essential nutrient with numerous associated health benefits (Block, 1991; Kalt, 2001; Olsson, 2004). Its well-known antioxidant activity is due to the ease with which it loses electrons thus acting as a reducing agent for a number of reactive oxidant species (Klein and Kurilich, 2000; Prior and Cao, 2000). Ascorbic acid also helps maintain enzyme configurations in the correct reduction state for catalytic activity, as well as having an involvement in deactivation of carcinogens (Kalt, 2001).

Ellagic acid is a naturally occurring polyphenolic secondary metabolite that accumulates in strawberry and raspberry fruits (Häkkinen *et al.*, 1999; Vatter and Shetty, 2005). The concentration of ellagic acid is generally high in strawberry, ranging from 0.16 to 2.07 mg g<sup>-1</sup> d. wt (Williner *et al.*, 2003). Most of the ellagic acid in plants is present within the vacuoles, as water-soluble ellagitannins rather than as the free acid. The biosynthesis of ellagic acid is thought to proceed via gallic acid. By conjugation with carbohydrates, gallic acid units form gallotannins, and the coupling of galloyl units in gallotannins is believed to produce the hexahydroxydiphenic units of the ellagitannins (Seigler, 1998). These ellagitannins release free ellagic acid upon hydrolytic cleaving.

Evidence for this biosynthetic scheme has been provided by Niemetz and Gross (2003, and see references therein) from studies of *Tellima grandiflora* leaves. Both free ellagic acid and ellagitannins (see Fig. 1) have properties which suggest they can have positive effects on human health (phytonutrients). Ellagitannins and ellagic acid, like other phenolics, have been shown to significantly contribute to the antioxidative activity of red raspberries and other fruits (Kalt, 2001; Mullen *et al.*, 2002; Vatter and Shetty, 2005). More recently, ellagic acid has been linked to health benefits by providing antimutagenic and anticarcinogen responses (Mandal and Stoner, 1990; Maas *et al.*, 1991a; Clifford and Scalbert, 2000; Perchellet *et al.*, 2002; Olsson *et al.*, 2004;

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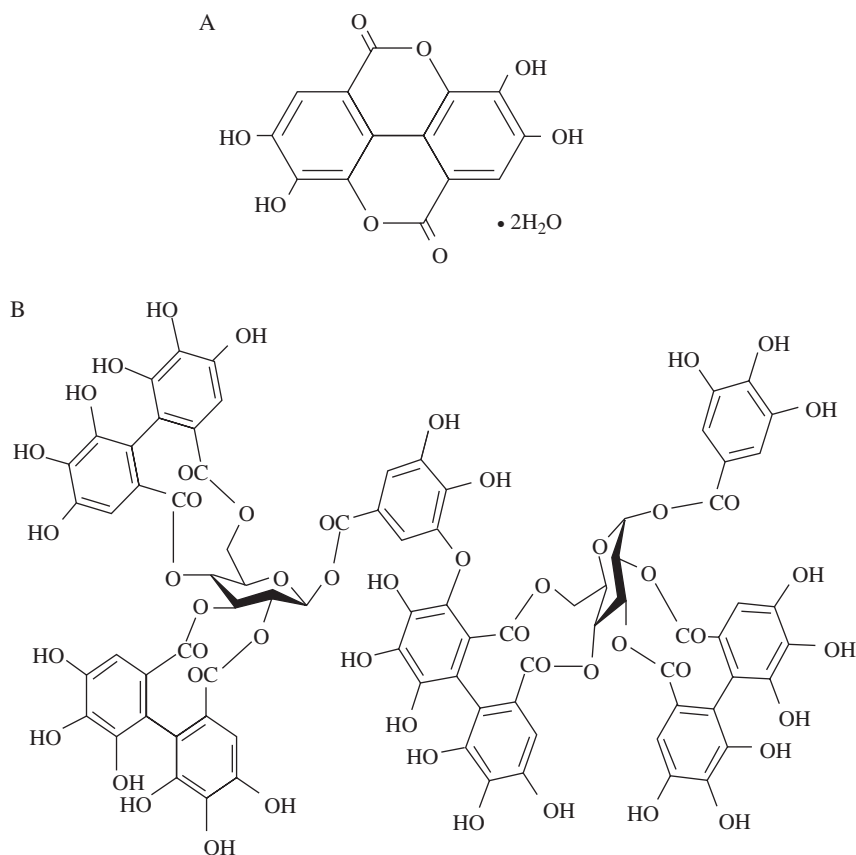


FIG. 1. The chemical structures of ellagic acid (A) and a sugar-conjugated ellagitannin (B).

Vattem and Shetty, 2005). These are important characteristics considering current efforts to improve dietary habits and minimize cancer risks (Williner *et al.*, 2003). However, the bioavailability of ellagic acid and ellagitannins is not well understood, and the potential anticancer and toxic effects need further study (Clifford and Scalbert, 2000).

Studies show that the concentration of ellagic acid in strawberry fruit varies 10-fold with cultivars (Maas *et al.*, 1991b) and declines with season; most of the ellagic acid in ripe fruit is present in the achenes (Maas *et al.*, 1991b; Williner *et al.*, 2003). Although little is known about European strawberry cultivars, there is evidence that fruit ellagic acid concentrations can be influenced by environmental conditions. Wang *et al.* (1998, 2002) showed that different mulch types had a significant effect on the concentration of ellagic acid in strawberry fruit, while Häkkinen and Törrönen (2000) showed an effect of geographical origin and cultivation technique on fruit phenolic acids in strawberries.

The development of agronomic techniques to enhance production of useful secondary metabolites provides an opportunity to optimize currently available cultivars. Furthermore, such agronomic manipulations (e.g. the application of mild stress at defined points during the growing season) may have generic effects on a range of phytochemicals.

The initial aim was to carry out a survey of conjugated and free ellagic acid concentrations in a range of strawberry

varieties grown in the UK. Total ellagic acid, i.e. free ellagic acid + conjugated ellagic acid, assumed to be released from ellagitannins, was measured. Also explored was the ability to enhance fruit ellagic acid concentration by altering crop load (number of fruit per plant). Manipulation of crop load can change the partitioning of resources, in particular the berry-to-berry competition for polyphenols such as ellagic acid. Stopar *et al.* (2002) showed that polyphenol concentration in *Malus* (apple) was increased by reducing crop load, although there was no effect of thinning on flavonoid and chlorogenic acid concentrations (Awad *et al.*, 2001).

Finally, the aim was to determine whether ellagic acid and ascorbic acid concentrations of two strawberry cultivars (a short-day or June-bearer and a day-neutral or everbearer) can be enhanced through the use of light (photosynthetically active radiation, PAR) reflective mulches. These mulches are believed to enhance light interception; this may improve crop photosynthesis, solar heat transmission, nutrient uptake, soil moisture conservation, root and flower development, fruit set, fruit development, fruit maturity, fruit quality (colour), yield and disease control. Work with apple has shown the effectiveness of reflective films in increasing light penetration into the canopy, and this correlates with increasing anthocyanin concentration in fruit peel (Ju *et al.*, 1999). Red colour development, at least in apple, is well documented as being light regulated (Siegelman and Hendricks, 1958;

Lancaster, 1992). Several key enzymes (including UDPGalactose:flavonoid-3-glucosyltransferase, UFGalT) have been shown to be light inducible (Saure, 1990; Ju *et al.*, 1995). Commercial strawberry production varies in its sophistication, but use of mulches (non-reflective) is extremely common. Reflective mulches offer potential practical benefits as well as the enhancement of bioactive compounds within fruits.

## MATERIALS AND METHODS

### *Strawberry cultivars used to survey ellagic acid concentration*

Fruit was collected at commercial ripeness from 45 different cultivars of field-grown strawberry plants in June–July (year 1) and from 17 cultivars in June–July (year 2), using plants from the breeding programme at East Malling. For each cultivar, several fruit (same hierarchical position) were collected from each of six separate plants to generate a representative pooled fruit sample. The fruit were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

### *Manipulation of strawberry fruit number (expt 1)*

Potted strawberry plants of the June-bearers ‘Elsanta’ and ‘Florence’ (R. W. Walpole Ltd, Norfolk, UK) were grown in a glasshouse. Four treatments were applied to each cultivar, and all treatments were replicated once in each of five blocks, with one replicate consisting of five plants. Plants were arranged according to a complete randomized block design. The treatments involved removing primary, secondary or tertiary flowers, as described below:

- Treatment (flower sample)—fruit samples taken
- Control (no flowers removed)—primary, secondary and tertiary berries
- Primary flower removed—secondary and tertiary berries
- Primary and secondary flowers removed—tertiary berries
- All flowers except the primary removed—primary berries

### *Manipulation of strawberry environment through the use of reflective mulches (expt 2)*

Two experiments were carried out, one with the June-bearing cultivar Elsanta, and a second with the everbearer cultivar Flamenco. Bare-rooted material (Darby Plants Ltd, Norfolk UK) was planted in commercial growing bags. Prior to planting, reflective mulches were applied to provide the following treatments:

- Treatment 1 = hessian (commercial brown weave = ‘zero reflectance’)
- Treatment 2 = black polythene
- Treatment 3 = Extenday™ (ST200, J & D Wilkie Ltd)
- Treatment 4 = Ultra-reflective (‘aluminized polythene’)

A  $1\text{-m}^2$  sheet of the relevant mulch material was positioned over the top of each growing bag, with the overlapping edges clipped on each side to wires, suspended approx. 20 cm above the ground. This ensured that the

mulch received any solar radiation that passed through the canopy. Once the mulches were in place, the bags were planted, on 3 Jun. 2003, at a commercial density of ten plants per bag.

Liquid fertilizer was continuously injected into the irrigation system using a dosatron. Standard Agrosol (Scotts UK Ltd) feed ( $1\text{ kg dm}^{-3}$  water). For the first month, Agrosol 313 (14:7:14, N:P:K) was used, switching to Agrosol F316 (13:5:28, N:P:K) once flowering had begun. The initial fertigation (nutrition and water given together) regime was 500 mL per bag (using three drippers, pressure-regulated to  $2\text{ L h}^{-1}$ ) four times daily (0800, 1200, 1600 and 2000 h). In early July, this was increased to 1000 mL, four times daily, to compensate for higher plant water usage. This continued until mid-September, when the fertigation was reduced to 500 mL per event. When it was necessary to adjust the fertigation in response to environmental conditions, i.e. during periods of high temperatures, an extra irrigation event was applied manually. Pollination was carried out manually with a fine brush.

Environmental conditions in the polytunnel and treatment differences were logged (Data logger DL2E, Delta T Devices, Cambridge, UK) throughout the season. For all treatments, temperature probes (thermocouples for air temperature and thermistors in the compost) were mounted in three positions: on the mulch surface; directly under the mulch; and in the compost at a depth of 5 cm. The thermocouple on the mulch surface was not exposed to direct radiation; temperatures recorded here were expected to decline as the plant grew and shading increased. For each treatment, temperatures were meaned ( $n = 3$ ) across three experimental blocks. Absolute PAR was continuously recorded with a series of quantum sensors mounted at fixed positions in the crop. All data were recorded at 1-min intervals and means logged over 30 min.

### *Spectral reflectance characteristic of the mulches*

Spectra of mulch-reflected light were measured between 280 and 800 nm with a double-monochromator spectroradiometer (Model SR99-1 PC; Macam Photometrics, Livingstone, UK) equipped with a 10-cm-diameter integrating sphere. The light source was a 75-W xenon-arc lamp (LOT Oriel, Leatherhead, Surrey). Reflectance was measured against a Spectralon white standard (LOT Oriel). Normalized data show the spectral balance of reflected light independent of total reflectance.

### *Seasonal in situ measurements of mulch reflectance*

Measurements of PAR were also used to determine seasonal changes in vegetative growth, using a multi-sensor (80 sensors) PAR ‘Ceptometer’ (AccuPAR, Decagon Devices Inc., USA). Records were made manually in the crop canopy at fortnightly intervals. For each bag, PAR readings were taken from three different positions: (1) incident radiation above the canopy, the probe held 0.5 m above the ground along the length of the bag; (2) incoming canopy radiation, the probe positioned in the leaf canopy, along the bag; and (3) radiation reflected from the mulch into the canopy, the probe held in same position as for (2) but

with the sensors inverted. For (2) and (3), the probe was held in the same position in respect to the mulch surface and the growing bag for each sampling date. Reflected measurements are expressed relative to the incident radiation; on each measurement occasion five replicate blocks per treatment were measured and the treatment average calculated.

#### *Experimental design*

The polytunnel was divided into two, with each cultivar occupying half. In both halves, a randomized block design was used, with each experiment comprising five blocks (replicates), running east–west, arranged in four rows running north–south. Each block contained one bag per mulch type, i.e. each experiment contained 20 bags: five blocks  $\times$  four treatments. One growing bag represented one experimental analysis unit.

#### *Developmental records*

Non-destructive measurements of vegetative growth were made from leaflet counts carried out fortnightly, using two plants per bag. All leaves that were judged to be at least 75 % fully expanded were counted. Runners were removed when appropriate and records were made of the total number collected per bag.

Leaf nitrogen concentration under optimal light conditions can be linked with plant growth potential; it can also be closely correlated with leaf chlorophyll concentration. Therefore, leaf ‘nitrogen content’ was monitored by measuring chlorophyll fluorescence (CCM-200, Opti-Sciences, USA) (to avoid nitrogen deficiency). Measurements were made fortnightly, using the youngest fully expanded leaf.

Volumetric measurements of compost moisture content ( $\text{m}^3 \text{m}^{-3}$ ) were recorded manually to ensure water availability was sufficient and similar for all the different treatments, irrespective of the impact of different compost temperatures. Measurements were made using a ThetaProbe connected to a moisture meter (type HH2; Delta T Devices, Cambridge, UK) using the manufacturer’s calibration for organic compost; taking three readings per bag, at fortnightly intervals.

#### *Destructive analysis of dry matter partitioning*

In early November, destructive records were made of the above-ground parts of ‘Flamenco’ plants; because ‘Elsanta’ ceased fruiting in August no analysis was carried out. The analysis involved counts of runners, fruits and trusses, as well as recording crown number and diameter, lamina area and fresh weight, petiole number and length, and dry weights of all plant parts.

#### *Crop records and fruit quality measurements*

Ripe fruit were harvested and size graded at least twice weekly from expt 2 until the end of October. Harvested fruit, per bag, was graded into four diameter size classes, i.e.  $>35$  mm, 25–35 mm, 22–25 mm, and waste (includes any small, diseased, rotten and malformed fruit). The largest two

classes constitute commercial class-1 fruit, with class-2 fruit being 22–25 mm. Once a week, fully ripe, medium-sized fruit were used to determine juice soluble solids (% Brix) with a digital refractometer (Palett 100, Atago & Co. Ltd, Tokyo, Japan).

Fruit samples were collected for chemical analysis (ellagic acid and ascorbic acid concentration) on several occasions during the growing season but, for comparative purposes, samples of ‘Elsanta’ and ‘Flamenco’ fruit harvested in mid-July are presented. Approximately 50-g samples of medium-sized ripe fruit were collected from each bag prior to being frozen in liquid nitrogen and stored at  $-20^\circ\text{C}$  until required for analysis as described below.

#### *Extraction and analysis of free and conjugated ellagic acid*

Approximately 10 g of frozen fruit was macerated in 50 mL of cold methanol:water (80:20 v/v) containing  $20 \text{ mg L}^{-1}$  butylated hydroxytoluene as an antioxidant. The macerate was centrifuged at  $1000g$  for 10 min. The supernatant was collected and its volume measured. For free ellagic acid determination, 5 mL of the supernatant was passed through a Waters C18 (Milford, MA, USA) solid phase extraction cartridge pre-equilibrated with methanol:water (80:20 v/v). This rp C18 was used as a ‘filter’ to remove non-polar co-extractives (see manufacturers’ guides, etc.) demonstrated by a high level of sample coloration retained on the C18. All C18 were conditioned with methanol and pre-equilibrated with 80 % methanol. A 2.5-mL aliquot of the eluate was reduced to dryness in a centrifugal vacuum concentrator and redissolved in 0.8 mL methanol:water (20:10 v/v). For total ellagic acid determination, 3 mL of the supernatant was mixed with 1.8 mL of 3.9 M HCl aqueous solution, to give a final concentration of 1.5 M HCl in methanol:water (50:50 v/v). The mixture was capped in a glass reaction vial and heated at  $100^\circ\text{C}$  for 2 h to hydrolyse the ellagitannins. After cooling, 2.5 mL of the resulting hydrolysate was passed through a Waters C18 solid-phase extraction cartridge pre-equilibrated with water, after washing with methanol to remove the HCl and non-polar hydrolysis products prior to HPLC. Ellagic acid was eluted from the cartridge with 5 mL of methanol:water (80:20 v/v).

Ellagic acid was analysed using a Waters Alliance 2690 HPLC system with a Waters 996 photodiode array detector. HPLC conditions were as follows: 20  $\mu\text{L}$  injection volume, Phenomenex Luna C18  $3\mu\text{m} \times 150$  mm HPLC column fitted with a C18 guard cartridge,  $35^\circ\text{C}$  column temperature, and a flow rate of  $0.2 \text{ mL min}^{-1}$ . The mobile phase was (A) 0.8 % trifluoroacetic acid in aqueous solution at pH 1.5, and (B) acetonitrile, and the following gradient was used: 0–2 min, 90 % A and 10 % B; 2–12 min, linear increase to 60 % A and 40 % B; 12–20 min, 60 % A and 40 % B; 20–30 min, linear increase to 10 % A and 90 % B; 30–45 min, linear decrease to starting conditions, with a further 5 min equilibration. Ellagic acid was detected at 254 nm. Quantification was by external standard calibration curve, generated from ellagic acid standards (ellagic acid

dehydrate) purchased from Avocado Research Chemicals Ltd, Heysham, Lancashire, UK.

The efficiencies of the extraction methods used for free and total ellagic acid were tested by repeatedly extracting the berry samples as described. For a high yield cultivar ('EM1055WF') 86.3% of the free ellagic acid was present in the first extract, 11.7% was present in the second extract, and 2.0% was present in the third extract. For a low yield cultivar ('St William's') 97.1% of the free ellagic acid was present in the first extract, 2.9% was present in the second extract, and 0.0% was present in the third extract. The efficiency of the extraction method for free ellagic acid, based on results from both cultivars, was  $91.7 \pm 5.4\%$  (mean  $\pm$  s.e. of the mean).

Recovery of total ellagic acid was slightly less efficient than for free ellagic acid. For 'EM1055WF' 81.5% of the total ellagic acid was present in the first extract, 15.6% was present in the second extract, and 2.9% was present in the third extract. For 'St William's', 88.9% of the total ellagic acid was present in the first extract, 8.5% was present in the second extract, and 2.6% was present in the third extract. The efficiency of the extraction method for total ellagic acid was estimated to be  $85.2 \pm 3.7\%$  (mean  $\pm$  standard error of the mean).

#### Extraction and analysis of total (oxidized + reduced) ascorbic acid

Approximately 20 g frozen weight of strawberry fruit was homogenized in a blender with 10 volumes of 25 mM ammonium acetate buffer pH 4.0. The homogenate was centrifuged at 24 000 *g* in a cooled (4 °C) ultracentrifuge for 15 min, and the volume of the supernatant measured. A 3-mL aliquot was added to 300  $\mu$ L of a 5% trifluoroacetic acid aqueous solution and 30  $\mu$ L of a 500 mM solution of the reducing agent TRIS (2-carboxyethyl) phosphine hydrochloride and left at 4 °C for 30 min. Five hundred microlitres of the resulting solution were pipetted into a Whatman Mini-UniPrep HPLC vial incorporating a 0.45- $\mu$ m polypropylene filter. HPLC conditions were as follows: Varian Polaris C18-A 3  $\mu$  150  $\times$  2-mm column (C18 column with a polar embedded phase) fitted with a Metaguard 4.6-mm Polaris C18-A 3  $\mu$  guard cartridge; column temperature 30 °C; mobile phase 0.5% trifluoroacetic acid in aqueous solution (A) and methanol (B); gradient 0–10 min 100% A, 10–15 min ramp to 50% A + 50% B, 15–25 min hold at 50% A + 50% B, 25–30 ramp to 100% A, 30–45 min, equilibrate at 100% A; flow rate 0.2 mL min<sup>-1</sup>. The Waters Alliance 2690 HPLC chromatographic system was again used for the analysis. Detection of ascorbic acid was at 245 nm, and quantitative analysis was by means of an external standard curve.

#### Statistical analysis

All experiments used replicated (see tables and figures of individual replication and degrees of freedom) and randomized designs. All statistical analyses were carried out using ANOVA to calculate *F* probabilities and the standard error of the difference of the means (s.e.d.) for treatment effects within cultivars.

TABLE 1. Concentrations of ellagitannins, free ellagic acid and total ellagic acid in ripe strawberry fruit harvested from 45 different cultivars in June–July in year 1

Strawberry cultivar	Concentration ( $\mu$ g g <sup>-1</sup> frozen weight)			
	Total ellagic acid	Free ellagic acid (EA)	Ellagitannins (ET)	Ratio ET:EA
Osmanli*	341	11.3	330	29.2
Nida	322	21.4	301	14.1
Laura	243	21.1	222	10.5
EM894-1	230	ND	ND	ND
Florence	226	ND	ND	ND
ITA93-971-59	218	12.7	206	16.3
Cigoulette	217	6.7	210	31.3
ITA91-355-3	208	7.9	200	25.4
EM676WF*	206	11.7	194	16.6
Jaune	201	5.0	196	38.8
Sophie	192	7.0	185	26.4
EM1088	190	11.8	178	15.0
EM1109WR	186	15.3	171	11.2
EM1089	181	16.2	165	10.2
Premial	177	8.0	169	21.0
Coral	176	23.0	153	6.7
EM726	176	ND	ND	ND
EM772-2	176	ND	ND	ND
EM835-2	174	ND	ND	ND
EM1097DC	166	6.2	160	25.7
Rosie	155	6.4	149	23.4
Emily	155	ND	ND	ND
Onda	154	3.8	151	39.2
Pandora	154	10.6	143	13.5
EM881-1	150	ND	ND	ND
Cigaline	149	12.9	136	10.5
Bolero	140	15.4	124	8.1
Darselect	139	4.1	135	33.1
Marshmello	135	ND	ND	ND
Pegasus	133	14.7	118	8.0
Selva	129	3.5	125	35.4
Ciloe	126	7.5	118	15.7
Symphony	124	10.9	113	10.4
Aromel	120	6.1	114	18.6
Eros	115	8.9	106	11.9
Cambridge Favourite	107	8.1	99.1	12.2
Totem	98.4	10.1	88.3	8.8
Elsanta	94.4	ND	ND	ND
Calypso	92.8	9.0	83.8	9.3
Korona	85.6	ND	ND	ND
Honeoye	84.4	10.9	73.5	6.7
Elvira	84.0	3.7	80.3	22.0
Tango	79.2	4.3	74.9	17.6
Hapil	60.0	ND	ND	ND

The cultivars are ordered by total ellagic acid concentration, in descending order.

ND, Not determined.

\* Cultivars which produce white fruit.

## RESULTS

### Strawberry cultivar variation in fruit ellagic acid concentration

Tables 1 and 2 show the range of concentrations of ellagic acid in 45 strawberry cultivars analysed in year 1, and in 17 cultivars analysed in year 2. Total ellagic concentrations varied considerably over the 2 years. Interestingly, the

TABLE 2. Concentrations of ellagitannins, free ellagic acid and total ellagic acid in ripe strawberry fruit harvested from 17 different cultivars in June–July in year 2

Cultivar	Concentration ( $\mu\text{g g}^{-1}$ frozen weight)			
	Total ellagic acid	Free ellagic acid (EA)	Ellagitannins (ET)	Ratio ET:EA
EM1055WF*	255	9.1	246	27.0
Florence	250	8.2	241	29.6
Symphony	239	12.6	227	18.0
EM1107WF*	236	5.7	230	40.3
Alice	235	10.1	225	22.3
Honeoye	232	11.2	221	19.8
Mira	230	18.5	211	11.4
Elsanta	223	8.1	215	26.7
Ciloe	205	5.8	199	34.6
Darselect	194	5.5	188	34.1
Evangelina	191	ND	ND	ND
Cambridge Favourite	187	9.8	177	18.0
Osmanli*	165	9.7	155	16.0
Oka	155	ND	ND	ND
Sophie	138	9.0	129	14.4
Onda	134	4.4	129	29.1
St William's	102	5.5	96.1	17.4

The cultivars are ordered by total ellagic acid concentration, in descending order.

ND, Not determined.

\* Cultivars which produce white fruit.

white-fruited cultivars ('Osmanli', EM676WF, EM1055WF and EM1107WF) were generally all within the upper ranges, although the individual cultivar rankings differed between the years. For example, the white-fruited 'Osmanli' had highest total ellagic acid in year 1 ( $341 \mu\text{g g}^{-1}$ ). In year 2, however, 'Osmanli' had only  $165 \mu\text{g g}^{-1}$  of total ellagic acid compared with  $232 \mu\text{g g}^{-1}$  in 'Honeoye'. This apparent year-to-year difference between cultivars was also evident with 'Symphony', 'Cambridge Favourite' and 'Elsanta'.

#### Effect of crop load on berry ellagic acid concentration

Tables 3 and 4 show that manipulation of crop load had no statistically significant effect on fruit ellagic acid concentration in either of the cultivars tested, either between treatments or berry types within a treatment. The greatest changes were seen for treatment 4 (secondary and tertiary inflorescences removed), where the primary berries had approx. 14% and approx. 10% less total ellagic acid than control primary berries in 'Elsanta' and 'Florence', respectively. However, these differences were not statistically significant.

As the concentration of ellagic acid was not affected by treatment, the variation of total yield of ellagic acid (averaged over all berry types) with treatment was similar to the variation of total fruit harvest weight. Treatments 2–4 necessarily reduced total fruit numbers, and the mean total amount of ellagic acid per fruit was greater in treatments 2 and 4 than in the control (treatment 1), due to an increase in average fruit size.

#### Effects of reflective mulches on the growing environment

Combined temperature data, from both cultivars, collected from above or below the mulch and in the compost are summarized in Fig. 2. These data show the average maximum and minimum temperatures from June to November. Generally, as seasonal temperature declined, treatment differences were reduced. Temperatures directly above the mulch, which are most likely to impact on above-ground growth, were increased by the black polythene, while both reflective mulches showed lower early season temperatures directly below the mulch relative to the hessian and black polythene. Compost temperatures showed similar seasonal trends, but the differences in treatment mean maximum and minimum temperatures were less marked. However, daily compost temperature fluctuations showed distinct differences, both with and between treatments, particularly with respect to the black polythene: this resulted in warmer compost during the day, but lower night-time temperatures relative to the other treatments (data not shown).

To determine the impact of compost temperature on water availability for growth, compost moisture content, measured from July to November, ranged from 0.40 to  $0.65 \text{ m}^3 \text{ m}^{-3}$  for 'Elsanta', and 0.45 to  $0.58 \text{ m}^3 \text{ m}^{-3}$  for 'Flamenco'; there was no consistent treatment effect (data not shown).

#### Measurements of mulch reflectance spectra

Reflectance spectra of the three plastic mulches were measured relative to the maximum reflectance for any film, in this case the ultra-reflective film (data not shown). Reflectance spectra are shown relative to each specific film (Fig. 3) to isolate the 'shape' of the reflectance spectrum from any difference in total reflectance. The Extenday and black polythene mulches have lower total reflectance than the ultra-reflective mulch, with a spectrum that is flat across the whole of the PAR and into the near infra-red. There is a progressive decline in reflectance with decreasing wavelength in the ultra-violet. For the ultra-reflective mulch, with its higher total reflectance, the spectrum is flat from near infra-red, through the PAR into the ultra-violet. There is, however, a sharp drop-off in reflectance below approx. 330 nm.

Figure 4 shows the reflected PAR measured *in situ* during the growing season. To exclude daily and seasonal changes in absolute PAR, measurements were expressed relative (as a percentage) to PAR above the crop (the external sensor). For both 'Elsanta' and 'Flamenco', the incident radiation declined during the season as the plant canopies expanded and intercepted a greater proportion of the PAR. Different seasonal changes were apparent with the two cultivars, due to initial differences in plant size, and the subsequent development patterns of June bearers and everbearers. More important were the treatment effects on PAR levels reflected back from the mulch. As well as marked seasonal differences, there were strong treatment effects on reflected PAR with the ultra-reflective and Extenday mulches reflecting 10–25% of the incident radiation in early summer, depending on cultivar. Initially, the smaller 'Flamenco' plants intercepted less incident PAR.

TABLE 3. Effects of crop thinning treatments on strawberry fruit ellagic acid concentrations

Cultivar and treatment*	Fruit type	Total ellagic acid concentration ( $\mu\text{g g}^{-1}$ )	Total fruit f. wt (g)	Total ellagic acid yield (mg)	Total number of berries produced	Estimated mean ellagic acid (mg) per fruit
'Elsanta'	1. Primary	112 <sup>†</sup> ± 13 <sup>‡</sup>	71 ± 25	7.4	5.3	1.4
	Secondary	106 ± 17	111 ± 13	12.2	11.0	1.1
	Tertiary	96 ± 20	107 ± 14	10.4	14.3	0.7
	Overall totals (or mean)	(102)	289	30.0	30.7	1.0
2.	Secondary	96 ± 14	83 ± 11	8.3	5.7	1.4
	Tertiary	101 ± 32	46 ± 30	3.9	4.7	0.9
	Overall totals (or mean)	(91)	129	12.2	10.3	1.2
3.	Tertiary	92 ± 12	48 ± 26	3.9	4.3	0.8
4.	Primary	96 ± 15	92 ± 16	9.2	5.7	1.6
'Florence'	1. Primary	209 ± 18	111 ± 18	23.7	8.3	2.8
	Secondary	198 ± 28	86 ± 24	15.9	10.0	1.6
	Tertiary	200 ± 37	13 ± 9	3.3	1.7	1.7
	Overall totals (or mean)	(203)	210	42.9	20.0	2.1
2.	Secondary	206 ± 22	67 ± 26	13.6	5.3	2.5
	Tertiary	209 ± 16	13 ± 8	5.3	1.5	1.8
Overall totals (or mean)	(205)	75.4	15.4	6.3	2.3	
3.	Tertiary	197 ± 23	19.0 ± 12	4.1	2.7	1.4
4.	Primary	188 ± 24	150 ± 24	29.2	7.7	3.7

n.s., Non-significant.

\* Treatments: 1, control—no flowers removed; 2, primary flowers removed; 3, primary and secondary flowers removed; 4, all flowers except primary removed.

<sup>†</sup> Total ellagic acid concentration is expressed as  $\mu\text{g g}^{-1}$  frozen weight of fruit; total ellagic acid yield is estimated by multiplying the concentration by the total fresh weight (g) of the berries harvested. Data for primary, secondary and tertiary berries are the means of three replicates. For treatments 1 and 2, an overall treatment mean taking into account the total harvest for all berry types has been calculated.

<sup>‡</sup> Means are shown plus 1 s.e.m.

TABLE 4. Statistical analysis of total ellagic acid concentration ( $\mu\text{g g}^{-1}$ )

Fruit analysed	Treatment*				Cultivar <i>F</i> probability	Cultivar s.e.d. (d.f.)	Treatment <i>F</i> probability	Treatment s.e.d. (d.f.)
	1	2	3	4				
Primary	+	.	.	+	<0.001	12.69 (6)	n.s.	20.06 (6)
Secondary	+	+	.	.	<0.001	14.79 (6)	n.s.	23.38 (6)
Tertiary	+	+	+	.	<0.001	16.52 (9)	n.s.	26.12 (9)

+, Fruit included in the analysis.

Cultivar × treatments interactions were not significant.

\* Treatments: 1, control—no flowers removed; 2, primary flowers removed; 3, primary and secondary flowers removed; 4, all flowers except primary removed.

Seasonal measurements showed that leaf 'chlorophyll content' increased for both 'Elsanta' and 'Flamenco', from approx. 12 to 25 units during July to November, but there were no treatment differences (data not shown).

#### Effects of reflective mulches on the distribution of dry matter within 'Flamenco' plants

Several plant organs showed statistically significant treatment differences in dry matter distribution (Table 5). Total petiole length, lamina area and fresh and dry weights, were increased by the ultra- and Extenday reflective mulch treatments. These effects were greatest for the Extenday mulch treatment. All of the other parameters measured indicated a greater, albeit not significant, allocation of

dry matter and total biomass to plants within the Extenday treatment.

#### Effects of reflective mulches on leaf growth of 'Elsanta' and 'Flamenco'

To determine dynamic changes in vegetative growth, frequent counts of leaf number were made. Initially, 'Flamenco' plants were smaller (approx. 15 leaves) relative to the 'Elsanta' (approx. 21 leaves) (Fig. 5). However, 'Flamenco' grew vigorously throughout July and early August, with leaf production exceeding that of 'Elsanta' by late July. Despite cultivar differences in growth and development of leaf number there were no statistically significant treatment effects (Fig. 5).

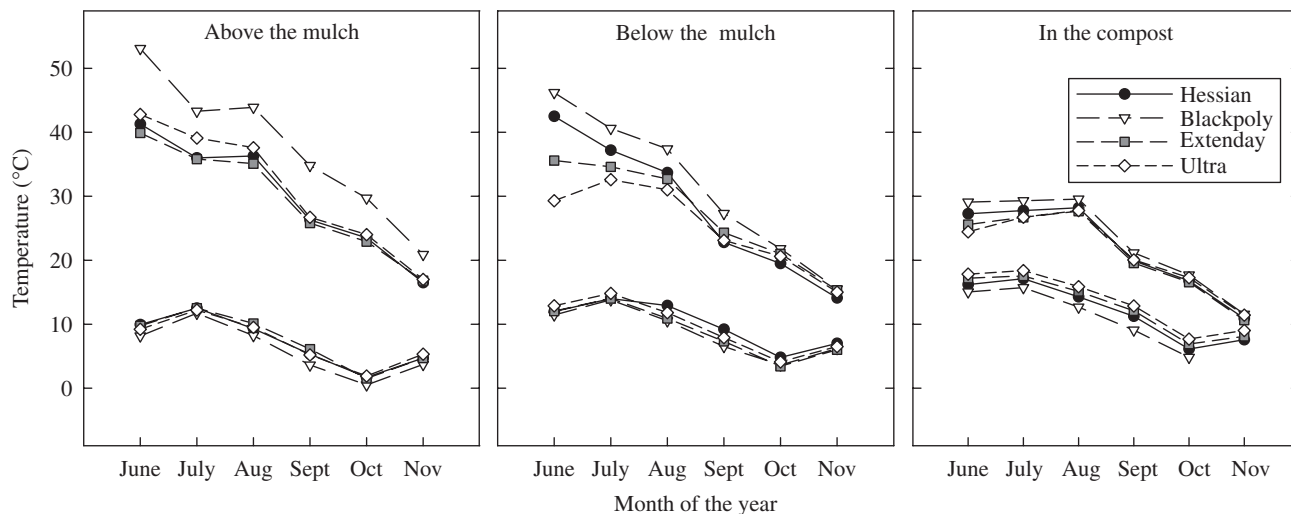


FIG. 2. The hourly mean average maximum and minimum temperatures for each month recorded throughout the growing season at three positions within the strawberry crop: air temperature above the mulch surface (5 cm); air temperature directly below the mulch on top of the compost; and soil temperature within the compost (at depth of 5 cm). Each data point represents data from three temperature sensors in three different experimental blocks. Data from both 'Elsanta' and 'Flamenco' are combined as there were no obvious cultivar differences.

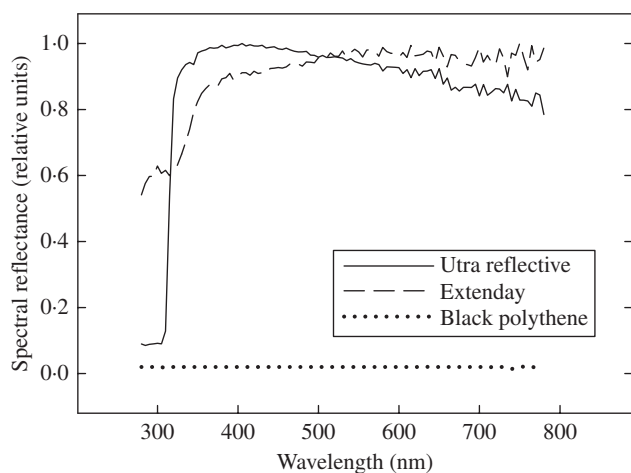


FIG. 3. Reflectance spectra of the three plastic mulches relative to the maximum reflectance achieved for each specific mulch. This shows the 'shape' of the reflectance spectrum irrespective of any differences in total reflectance between mulches.

*Effects of reflective mulches on productivity and seasonal cropping patterns of 'Flamenco' and the total crop weight and fruit number of 'Elsanta' and 'Flamenco'*

The effects of the reflective mulches on the seasonal cropping patterns for 'Flamenco' are shown, over a 17-week cropping season, in Fig. 6 (top). Mean weight of class-1 fruit is shown as an average weekly total harvested per bag, i.e. ten plants per bag. Seasonal variation in fruit harvested was considerable, with crop weight peaking at weeks 7 (18 Aug.) and 11 (15 Oct.). The early production peaks, particularly in week 7, were dominated by the Extenday and ultra-reflective treatments. By week 11, all treatments yielded similar crop weights and, from week 13, yields from the hessian treatment declined.

When expressed as cumulative seasonal crop yields for total class-1 fruit, and separately for the very large class-1 fruit (>35 mm), the phasic nature of the cropping season and the treatment trends were apparent (Fig. 6, bottom). By week 5 (4 Aug.) the ultra-reflective and Extenday treatments had the highest rates of production, while the black polythene treatment had the lowest. By week 11, despite a decline in cropping with all treatments, the ultra-reflective and Extenday treatments still exhibited the highest fruit production rates.

The greater total mean crop yield for the everbearer 'Flamenco' compared with the June-bearer 'Elsanta' was, as expected, due to the differences in cropping season length (Table 6). Treatment differences were also apparent, with ultra-reflective mulch significantly increasing the total fruit weight for 'Elsanta' (25%) and 'Flamenco' (16%), and fruit number for 'Flamenco' (19%). This increase in fruit production, for both cultivars, resulted from a significant increase in class-1 fruit (all fruit >25 mm in size) (Table 7).

*Effects of reflective mulches on seasonal soluble solids (Brix %) of 'Flamenco'*

Values for soluble solids were highest in mid-July but then declined during August and September, only to increase back to July values in October (data not shown). The seasonal trends were generally consistent, with the only significant difference being in early September when the fruit grown with ultra-reflective and Extenday mulches had higher soluble solids. There was a seasonal link between fruit production and soluble solids; when the former was highest, soluble solids were lowest.

*Effect of reflective mulches on fruit total ellagic acid and ascorbic acid concentration*

Mulch type affected ellagic acid concentration in 'Flamenco' but not in 'Elsanta' (Table 8). The Extenday



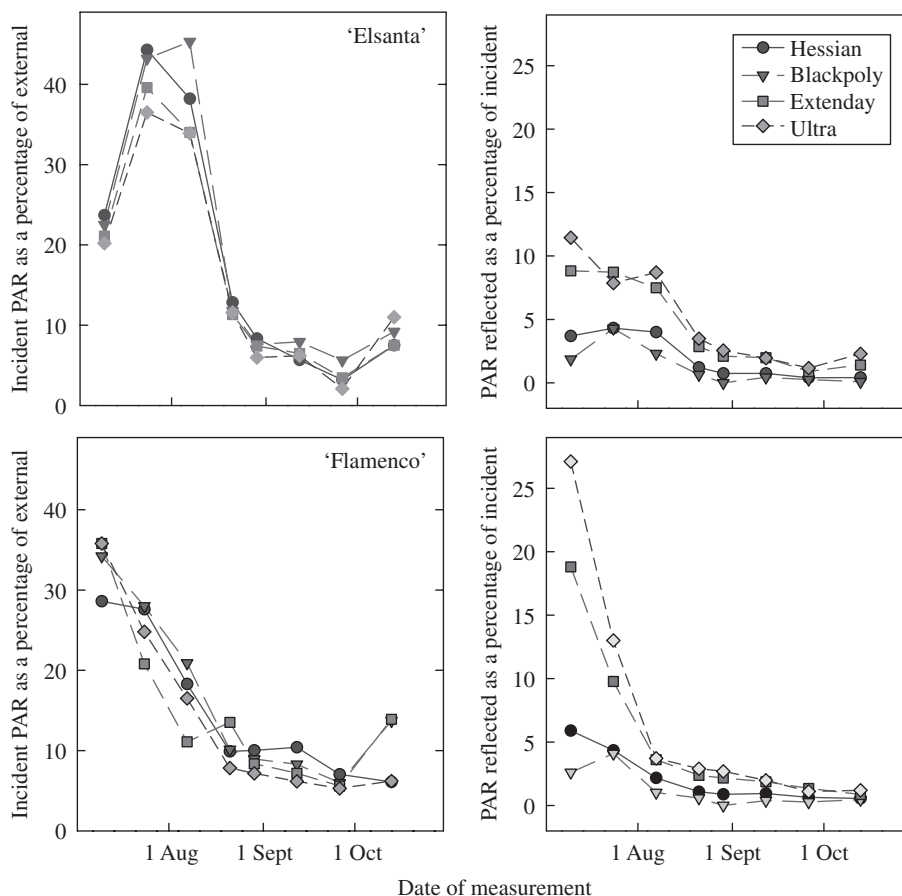


FIG. 4. The amount of incident radiation (PAR) detected close to ‘Elsanta’ (top) and ‘Flamenco’ (bottom) strawberry plants grown on a range of mulches with different reflective properties. Incident radiation is expressed directly in proportion to values of an external sensor to remove daily and seasonal changes during measurement. Reflected radiation is measured at the same position as the incident radiation, with the sensors inverted, and is expressed as a proportion of the incident radiation. Each data point represents the mean of four replicate blocks measured on each date.

TABLE 5. Analysis of plant dry matter distribution to crowns and leaves, and plant size for ‘Flamenco’ strawberries grown on a range of mulches with different reflective properties

	Mulch type				Statistical analysis		
	Hessian	Black polythene	Extenday™	Ultra-reflective	F probability	s.e.d.	d.f.
Crown number	54	52	64	48	0.05	5.26	12
Total crown diameter (mm)	507	454	573	464	0.074	44.3	12
Petiole number	328	334	380	350	0.284	27.6	12
Total petiole length (mm)	3554	3953	4538	3947	0.042	297.0	12
No. of non-fruiting trusses	33	33	39	40	0.728	7.6	12
Lamina area (cm <sup>2</sup> )	14734	15637	17687	16127	0.063	981.4	12
Lamina f. wt (g)	357	372	434	400	0.047	25.4	12
Lamina d. wt (g)	98	100	121	110	0.044	7.7	12
Total vegetative d. wt (g)	233	237	282	253	0.110	20.1	12

Plants were harvested at the end of the growing season (3 Nov.) and values are the means from 50 plants. All data are mean values per bag (i.e. total of ten plants).

polythene mulch significantly increased fruit total ellagic acid concentration in ‘Flamenco’ by 40 % over the hessian mulch ( $P = 0.03$ ). The ultra-reflective mulching increased ellagic acid concentration to a lesser extent: 20 % greater compared with hessian.

Table 9 shows that, in contrast to the results reported for total ellagic acid, mulch type affected ascorbic acid concentration in ‘Elsanta’ but not in ‘Flamenco’. The Extenday mulch increased fruit ascorbic acid concentration in ‘Elsanta’ by 11 % over hessian and by 14 % over black

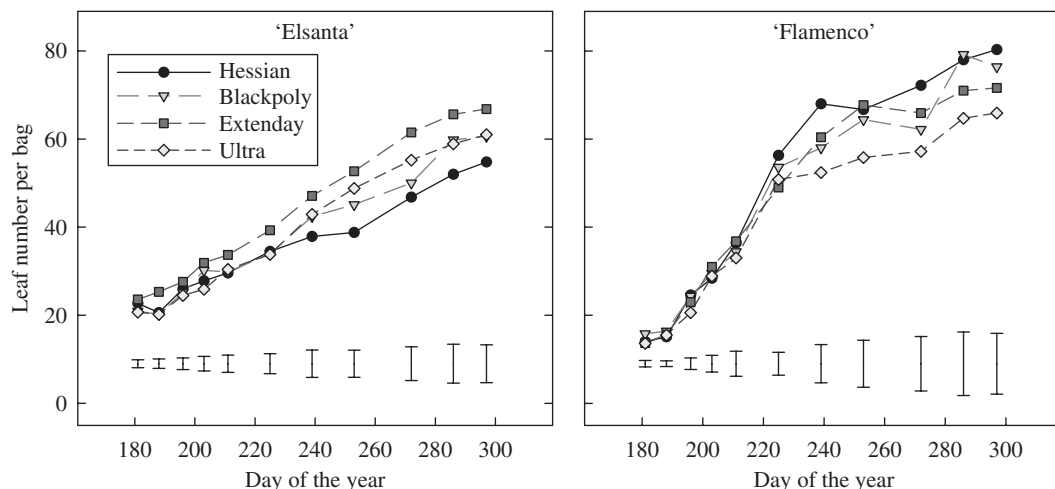


FIG. 5. Seasonal change (day of the year) in leaf number for 'Elsanta' (left) and 'Flamenco' (right) strawberry plants grown on a range of mulches with different reflective properties. The bars represent the s.e.d. for each measurement date.

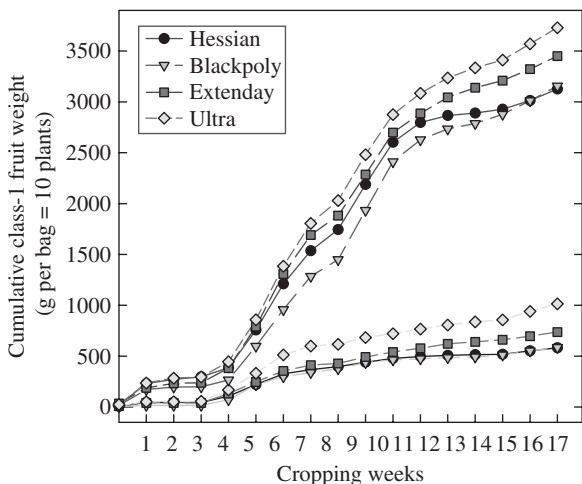
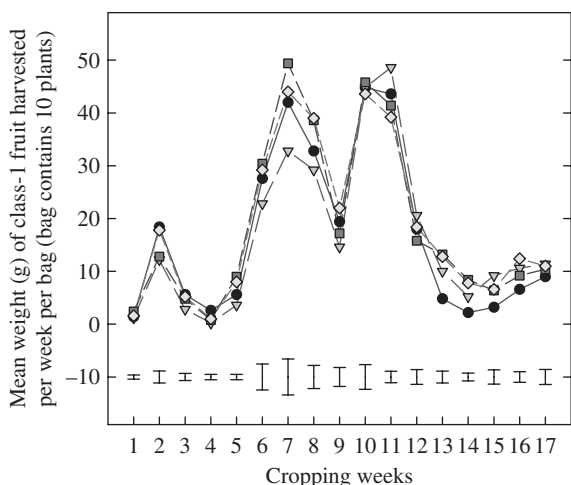


FIG. 6. Seasonal changes in the weight of class-1 fruit from 'Flamenco' strawberry plants grown with a range of mulches with different reflective properties. The mean average weight of class-1 fruit harvested per week per bag (ten plants) and the cumulative weight of total class-1 fruit harvested throughout the growing season (top right) and the >35-mm class-1 fruit (bottom right).

TABLE 6. Mean total season crop weight and fruit number harvested over the entire season from 'Elsanta' and 'Flamenco' strawberries grown on a range of mulches with different reflective properties

Mulch type	Elsanta		Flamenco	
	Fruit weight (g)	Fruit number	Fruit weight (g)	Fruit number
Hessian	1615*	207	4095	485
Black polythene	1465	192	4050	505
Extenday™	1698	202	4403	528
Ultra-reflective	2012	243	4759	575
F probability	0.025	0.199	0.05	0.033
s.e.d.	154	23.1	248.2	27.2
d.f.	12	12	12	12

\* Total average weight was from a bag containing ten plants.

TABLE 7. Mean crop weight (g) harvested per bag (ten plants) with respect to fruit size class for 'Elsanta' and 'Flamenco' strawberries grown on a range of mulches with different reflective properties

Mulch type	Elsanta			Flamenco		
	Class 1*	Class 2	Waste	Class 1	Class 2	Waste
Hessian	1282	270	10.6	3126	839	52.3
Black polythene	1168	265	13.2	3315	709	44.6
Extenday™	1435	215	15.8	3450	821	34.4
Ultra-reflective	1726	219	9.5	3729	855	36.2
F probability	0.006	0.355	0.384	0.02	0.447	0.149
s.e.d.	130.7	37.8	3.78	182.1	96.2	7.99
d.f.	12	12	12	12	12	12

\* Class 1, >25 mm; Class 2, 22–25 mm; Waste <22 mm and misshapes, etc.

polythene, and there was a significant treatment effect. Ultra-reflective mulching increased ascorbic acid concentration by slightly less than Extenday mulching (8% greater than for hessian and 10% greater than for black polythene).

TABLE 8. Total ellagic acid concentration in fruit from 'Elsanta' and 'Flamenco' strawberries grown on a range of mulches with different reflective properties

Mulch type	Total ellagic acid concentration ( $\mu\text{g g}^{-1}$ frozen weight)	
	Elsanta	Flamenco
Hessian	184*	137
Black polythene	172	154
Extenday™	179	191
Ultra-reflective	192	163
F probability	0.754	0.031
s.e.d.	19.4	16.0
d.f.	12	12

Fruit samples for analysis were taken in mid-July.

\* Data shown are the means of five replicate blocks.

TABLE 9. Total ascorbic acid (vitamin C) concentration in fruit from 'Elsanta' and 'Flamenco' strawberries grown on a range of mulches with different reflective properties

Mulch type	Total vitamin C concentration ( $\mu\text{g g}^{-1}$ frozen weight)	
	Elsanta	Flamenco
Hessian	725*	603
Black polythene	709	591
Extenday™	804	567
Ultra-reflective	781	607
F probability	0.038	0.567
s.e.d.	32.6	30.0
d.f.	12	12

Fruit samples for analysis were taken in mid-July.

\* Data shown are the means of five replicate blocks.

## DISCUSSION

The results from an initial survey of UK strawberry cultivars showed a large range in fruit total ellagic acid concentration—from 60 to 341  $\mu\text{g g}^{-1}$  frozen weight. The present results are in the same range as those of Maas *et al.* (1991b), who reported ellagic acid concentrations between 0.43 and 4.64 mg  $\text{g}^{-1}$  d. wt in different US-grown strawberry cultivars (roughly equivalent to 43–464  $\mu\text{g g}^{-1}$  f. wt assuming a 10:1 ratio of fresh weight: dry weight). Similarly, Häkkinen and Törrönen (2000) showed that ellagic acid concentrations in strawberries grown in Finland and Poland varied from 340 to 590  $\mu\text{g g}^{-1}$  f. wt. Williner *et al.* (2003), however, measured lower ellagic acid concentrations of 16–46  $\mu\text{g g}^{-1}$  f. wt in Argentine-grown strawberries. Differences between these results and those shown here, along with those of Maas *et al.* (1991b), may be due to the basis of data expression (fresh, dry and frozen weights), or perhaps the impact of cultural or climatic variation.

Berries from the white-fruited cultivars tended to be in the higher range of total ellagic acid concentration. Ellagic acid biosynthesis is believed to proceed from gallic acid, which is formed directly from shikimic acid (see Seigler, 1998; Niemetz and Gross, 2003). The biosynthesis of anthocyanins is also dependent on shikimic acid, although

they are downstream in the biosynthetic pathway. It is unclear where a blockage in anthocyanin synthesis occurs in these white-fruited cultivars, or if the lack of colour is linked to a direct or indirect effect on channelling precursor into ellagitannin formation. Shikimic acid itself does not accumulate at high concentrations in strawberry fruit; Sturm *et al.* (2003) measured a range of 6–18  $\mu\text{g g}^{-1}$  f. wt in ripe fruit. Preliminary investigations of ellagic acid concentrations in yellow or apricot-coloured raspberry cultivars, which were presumed to show a degree of anthocyanin-deficiency, did not show elevated ellagic acid concentrations (J. Le Mièrè and Y. Y. Ford, unpubl. res.).

Comparison of year-to-year variation in total ellagic acid concentration showed significant differences between cultivars. There were also considerable differences in the conjugated and free ellagic acid ratios for the different cultivars. Seasonal cultivar differences were principally caused by changes in conjugated ellagic acid. In all cases, the conjugated ellagic acid was present in greater quantities than the free acid, but the ratio of conjugated ellagic acid:free ellagic acid was not as great in strawberry as in raspberry, which had little free acid (J. Le Mièrè and Y. Y. Ford, unpubl. res.; Mullen *et al.*, 2002). Seasonal variation in total ellagic acid suggests that its concentrations may be environmentally influenced; this suggests the possibility of agronomic manipulation. Other researchers have reported this seasonal variation in antioxidant concentration (EU COST 863, 'Euroberry').

As yet, it is not known whether ellagic acid biosynthesis occurs in its entirety in leaves and fruit of strawberry, or whether ellagic acid accumulation in the fruit is partly or wholly dependent on the supply of precursors from leaves (shikimic or gallic acid, or precursors upstream). Shikimic acid concentration increases during strawberry fruit ripening, but the increase is small and does not affect the overall decrease in total acids (Sturm *et al.*, 2003). Ellagic acid concentration decreases during strawberry fruit ripening (Maas *et al.*, 1991b; Williner *et al.*, 2003), probably as a result of fruit cellular expansion (dilution). Manipulation of crop load, by flower removal, may alter berry-to-berry competition for leaf-derived assimilates. However, there was no significant effect of crop load on fruit ellagic acid concentration, and the total yield of ellagic acid per plant declined with severity of flower thinning. Thus, a decrease in fruit number per plant did not lead to an increased accumulation of ellagic acid in the remaining berries, suggesting that ellagic acid concentration is relatively tightly regulated in strawberry, irrespective of crop load.

The agronomic impacts of the two reflective mulches on fruit yield are important, particularly where the ultra-reflective mulch increased yields in the absence of enhanced vegetative growth (leaf number). Harvest costs are negatively influenced by increases in leaf canopy because the fruit are difficult to find. It is equally important when trying to enhance yields that fruit quality is not compromised; here reflective mulches enhanced fruit quality through an increased proportion of fruit in class 1. Fruit quality is not solely dependent on fruit size with several factors such as taste and texture also interacting.

Increased yields in response to the use of mulches and reflective mulches are not in themselves novel; many studies have documented increases in yield for a number of crops (Dufault and Wiggans, 1981; Farias-Larios and Orozco-Santos, 1997; Csizinszky *et al.*, 1999; Greer and Dole, 2003). However, improvements in crop quality are not often recorded and, as far as is known, this is the first study to describe dual benefits of reflective mulches on strawberry fruit yield and chemical composition. The type of mulch used in strawberry cultivation appears to have different responses with respect to the antioxidant altered and the cultivar used. Using reflective mulches significantly increased the concentration of total fruit ellagic acid (by between 20 % and 40 %) in 'Flamenco' and that of the ascorbic acid in 'Elsanta'.

The present results show that antioxidant capacity, with respect to ellagic acid concentration, varies with season, but as yet it is not known why. This phenomenon is not solely associated with these experiments; other researchers within Europe also report such variation (R. Nestby and E. Krüger-Steden, pers. comm.). More importantly, these results suggest that opportunities exist for developing agronomic practices to manipulate growth, to enhance yields and to optimize fruit bioactive compounds.

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