ANNUAL REPORT

To:
Horticultural Development Council
Bradbourne House
Stable Block
East Malling
Kent
ME19 6DZ

HDC Fellowship Scheme

April 2007

Commercial – In Confidence

Project title: HDC Research Fellowship Scheme

Project number: PC 221

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Report: Annual Report

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Date commenced: 1 March 2004

Duration: 3 years

The results and conclusions in this report are based on a series of crop scale observations, crop trials and more detailed field- and laboratory-based experiments. The conditions under which the studies were carried out and the results have been reported with detail and accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with the interpretation of the results especially if they are used as the basis for commercial product recommendations.

Authentication

I declare that this work was done under my supervision according to the procedures described herein and that this report represents a true and accurate record of the results obtained.

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List of contents

CP19 Horticultural crops: Further demonstration of the potential benefits of modified plastic crop covers report which forms 75% of the Fellowship is submitted separately.

PART ONE: Development of the method involved in extracting and assaying the activity of cell wall bound peroxidase activity in Iceberg lettuce (*Lactuca savita L*)

Plant material

Whole plants of Iceberg lettuce (*Lactuca savita L*) (Rijk Zwaan, The Netherlands) were harvested and fresh weight was determined for each sample. Leaves were then stored in liquid nitrogen for subsequent assay of cell wall associated peroxidase activity using a method based on that detailed in Bacon et al (1997), but modified for *Lactuca savita* on the basis of the following investigations.

pH optimum

The effect of a series of buffer and assay pH values between 5.5 and 7.0 were assessed for whole plant shoot tissue (Fig. 1). In this case pH 6.0 and pH 6.5 were both found to be the optimum. However, pH 6.0 produced reduced variability and therefore all buffer, extraction and assay solutions were corrected to this pH.

Optimal number of low salt washes required to remove cytoplasmic peroxidase activity

Samples were homogenised in liquid nitrogen before being transferred to ice cold buffer (10 mM sodium succinate, 10 mM calcium chloride, 1mM dithiothreitol, pH 6.0) (Sigma Poole, U.K.) at a ratio of 10:1 buffer to sample fresh weight. 200 µl of buffer and homogenised tissue was then centrifuged at 2000g for 5 mins. The pellet was washed four times in 200 µl of 10 mM sodium succinate to remove cytoplasmic peroxidase activity, before being re-suspended in an equal volume of final extraction buffer (50 mM sodium succinate, 1 M sodium chloride, pH 6.0) to extract activity from the cell wall. The four low ionic concentration buffer wash stages reduced

activity derived from tissue by c.95% (Fig. 2). The final high salt wash extract showed an increase in activity when compared to the final wash of the four low salt concentration washes (Fig. 2).

Measurement of cell wall peroxidase activity

Activity was determined by assaying a 100 µl sample of the supernatant using the guaicol method detailed by Chance & Maehly (1955). The 100 µl sample was added to 1 ml of 20 mM sodium phosphate buffer, which contained 276 µl of guaiacol (Sigma, Poole, Dorest, U.K.) per 50 ml of buffer. The reaction was started by adding 100 µl of 0.03% hydrogen peroxide in distilled water (w/w) (Sigma, Poole, Dorset, U.K.). The concentration of hydrogen peroxide and guaiacol used gave a linear change in absorbancy over 20+ minutes (Fig. 3). The reaction was mixed in 1.5ml spectrophotometric cuvettes (BDH, supplied by Merk Ltd, Lutterworth, Leicestershire, U.K.). The absorbancy of the solutions at 470 nm was then measured after 20 mins using a Cecil series 2 spectrophotometer at 25°C (Cecil instruments, Cambridge, U.K.).

a)

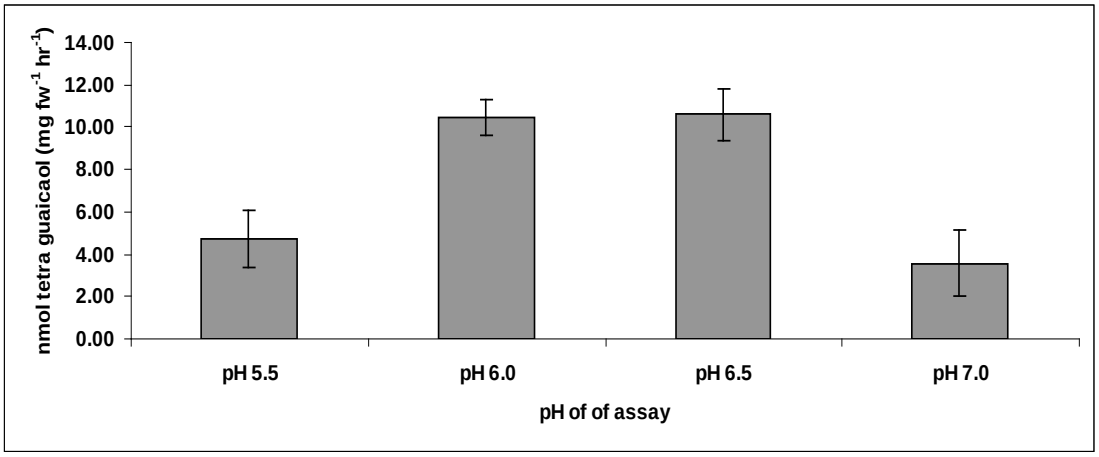


Figure. 1. Assay of extracted cell-wall associated peroxidise activity at different pH values in leaf 2 of *Lactuca savita L.* Each value is the mean of five determinations of activity ± S.E.

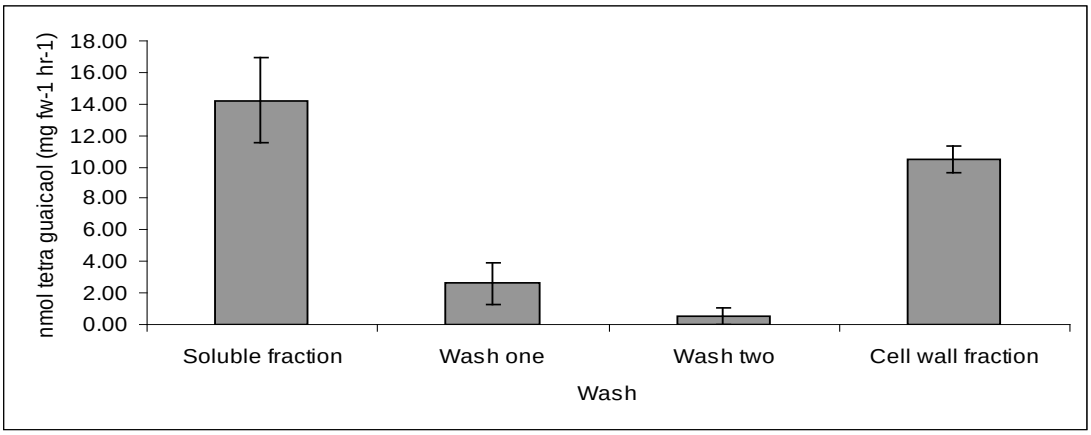


Figure. 2. Assessment of the ability of low and high salt washes to remove and recover (respectively) of both cytoplasmic (soluble fraction, wash one and two) and cell-wall associated peroxidase activity in leaf 2 of *Lactuca savita L.*Values are the mean of five determinations of activity ± S.E.

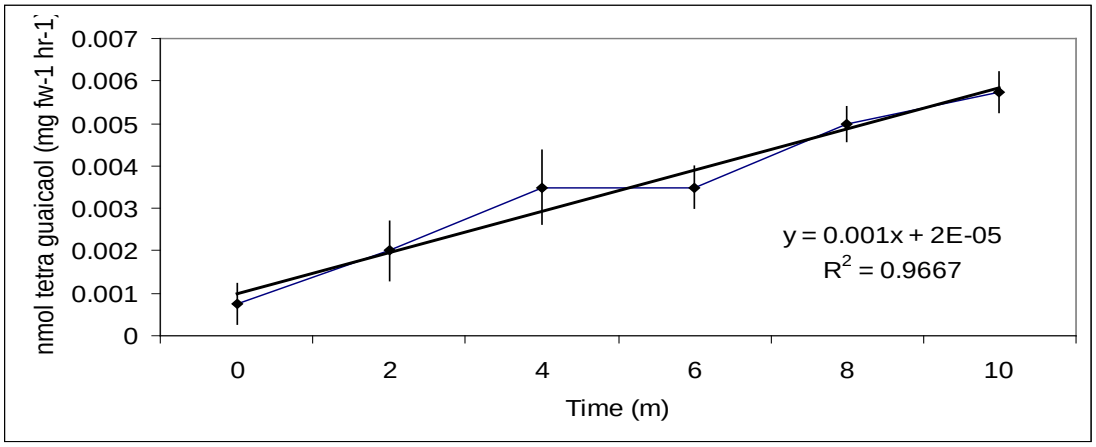


Figure. 3. Plot of change in absorbance over time demonstrating linearity of the assay using 100 µl of extracted activity 5.52µl of guaiacol and 100 ml of 0.003% hydrogen peroxide in 1ml of phosphate buffer. Each value is the mean of five determinations of activity ± S.E.

PART TWO: Modification of leaf morphology by altered solar UV under spectral filters mediated by cell wall peroxidases.

Summary

Crop plant growth responses to light spectral quality can be exploited to deliver a range of agronomically and economically desirable end-points. This can be achieved using a new generation of plastics with specific spectral properties as crop covers. Over a period of four UK growing seasons we have investigated the potential of the following three plastics a) a widely used commercial Standard clear b) a filter that is largely UV transparent and c) a UV blocking filter in manipulating propagation lettuce (*Lactuca sativa)* morphology to deliver these commercially desirable end-points. At the end of the propagation stage leaf area was significantly reduced and leaf thickness increased under UV-transparent compared to both the Standard (10%) and UV-opaque (30%) treatments. Final epidermal cell areas in leaf two of UV-transparent was reduced by approx. 10% and 19% when compared to Standard and UV-opaque respectively. Cell-wall bound peroxidase activity was measured in leaf two of all filter treatments over a 6 d period following leaf emergence. Activity increased immediately following emergence and remained elevated in Standard and UV-transparent when compared to UV-opaque for the duration of the experiment. In separate field experiments designed to investigate the effect of morphological changes at the propagation stage on crop productivity to harvest the UV-transparent propagated lettuce increased final yields by 23% compared to Standard and 15% relative to UV-blocking. This study suggests that propagating lettuce under a crop cover with high UV transmission properties induces peroxidise mediated in changes in leaf morphology leading to increased performance of the crop in the field.

Introduction

A reduction in leaf expansion is one of the most consistent responses to plant exposure to ultraviolet radiation (280-400nm). This is best defined for UV-B radiation (280-315nm), where both increases representative of those resulting from ozone depletion (F&C meta-analysis) and attenuation of ambient solar UV-B have been shown to reduce expansion in a wide range of species. In addition, the more limited literature on UV-A radiation (315-400nm) confirms that selective filtration of this waveband from sunlight also leads to increased leaf growth (Gonzalez et al. 1998).

As well as being a key response in terms of understanding ecological responses to UV radiation, control of leaf growth by these wavelengths may also have valuable practical applications for growth regulation in amongst others; propagation crops. There are been several attempts to exploit UV responses for crop growth regulation, both using UV lamps and, more recently, wavelength selective filters. Advances in these filters have allowed the manufacture of novel materials that 'fine-tune' the growing environment, by manipulating the intensity and wavelength of light reaching the crop. Specifically, plastics are now commercially available that either transmit 94% and 84% of UVA and UVB respectively up to 400 nm. In a number of Mediterranean countries filters that block all solar UV radiation from reaching the crop are now used commercially as an element of pest and disease control (Raviv & Antignus, 2004), but their effects on crop growth or morphology appear to be poorly defined.

Despite the consistent evidence that UV radiation reduces leaf expansion, the underlying mechanisms remain poorly understood. The growth and development of leaf tissue, a primary determinant of biomass production, is characterised by the integrated control of cell division, cell expansion and cell differentiation (Van Volkenburgh, Stahlberg & Bultynck 1998; Van Volkenburgh 1999). The dynamic character of the cell wall provides a mechanism(s) with which plants are able to selectively modify the extracellular matrix of different cell types, as a consequence of growth and differentiation, and in response to biotic stress (e.g. pests and disease) and changes in the abiotic environment (e.g. drought and elevated UV) (Cosgrove, 2001; Akiyama & Pillai, 2001; Peters, Hagemann & Tomos, 2000).

The physical control of cell expansion resides in the ability of the cell wall to respond to turgor-driven hydraulic pressure through the rearrangement or loosening of the existing cell wall (Fry 1986). These changes in the wall's mechanical properties correlate with changes in growth rates in several studies (e.g. Fry 1986; Zheng & Van Huystee 1992; Bacon, Thompson & Davies 1997). Therefore the properties of the cell wall are key components of leaf growth and can be modified by a number of cell-wall associated enzymes including peroxidases (Fry 1986).

Peroxidases (donor: H_2O_2 oxidoreductase; EC. 1. 11. 1. 7) are oxidoreductases that catalyze the oxidation of a wide spectrum of organic compounds using hydrogen peroxidase as the ultimate electron acceptor (Dawson 1988). Involvement of peroxidases in growth processes through cell wall rigidification is well documented (Bacon *et al*. 1997; Gaspar *et al*. 1985), and their activity is inversely related to growth inhibition mediated by plant hormones (Tse-Min & Yaw-Huei 1996). Specific roles for peroxidase activity have been established in decreases in plant growth rates (MacAdam, Nelson & Sharp 1992; Zheng & van Huystee, 1992), the assembly of lignin (Lagrimini 1991; Klotz *et al*. 1998), the linking of protein and lignin on the cell wall (Fry, 1986), its subsequent rigidification (Richard & Job, 1974; Gaspar *et al*. 1985) and in response to both biotic (Scott-Craig et al 1995) and abiotic ( ) stress.

The aim of this study is to examine the fundamental mechanisms through which leaf growth is affected by solar UV, using manipulation of solar UV by crop-scale spectral filters. We focus upon the use of plastic films with three contrasting UV properties for affecting changes in leaf development, investigating the underlying mechanisms driving those changes and subsequent field performance in propagation lettuce.

Materials and Methods

Plant material.

Plants of Iceberg lettuce (*Lactuca sativa* L. cv. Challenge, Syngenta seeds Ltd, UK) were raised for 14d from sowing using a widely employed UK commercial practice at Crystal Heart Salads (Holme-on-spalding-Moor, UK). Briefly, seeds were germinated in 4cm^3 peat blocks (Fison B2 Blocking Compost, Fisons, UK) at 16 $\pm$ 3°C in the dark for 4d before being transferred to commercial glass for a further 10d. At 14d plants were transferred to STC and randomly distributed under the four filter treatments for a period of 14d at which completed the propagation stage.

*Crop-scale experiments: The facility at Stockbridge Technology Centre***.**

All crop-scale experiments were carried out at Stockbridge Technology Centre (STC: 53N 1W) using a series of commercial high-tunnel structures (Haygrove Tunnels Ltd., Ledbury, UK). Each spectral filter structure covers 740m^2 over four individual bays, each measuring 3 m high X 6 m long.

Plastics.

In our experiments we make use of a range of three commercially produced plastic cladding films (all supplied by Bpi.agri Ltd., Stockton-on-Tees, UK). In all cases the base film is 150-µm-thick polyethylene, with specific additives, conferring specific spectral transmission properties. The control film (Standard) is a standard commercial horticultural cladding film that had a PAR transmission of 93% when new. Transmission in the UV declines rapidly with decreasing wavelength from 90% at 400

nm to less than 10% below 350 nm. Total UV-A transmission is approximately 50%. Transmission in the UV-B is less than 5% and effectively zero below 300 nm. Two films with modified UV transmission are used. The UV-opaque film has a total PAR transmission of 95% but a total UV-A transmission of only 10% and its UV-B transmission is zero. Transmission in the UV is zero below 375 nm but increased to around 60% at 400nm. The UV-transparent film has a transmission greater than 80% across the whole of the solar UV range from 290 to 400 nm. Total transmission in the PAR and the UV-A are 94% and 90% respectively.

Leaf expansion measurements.

Daily expansion was measured from the time of leaf emergence. Both length and width measurements of leaf 2 were taken at the widest point using electronic digital callipers and area calculated accordingly (Screwfix Direct, Yeovil, UK). Daily area growth increments, which correlate highly with absolute leaf area (r^2 = 0.97 - 0.99, depending on species), were calculated from lengths and widths, measured using a LI-3100 area meter (LI-COR Inc, Lincoln, Nebraska, USA) at destructive harvests throughout development. In instances where destructive harvests were made determination of leaf area was made using an automatic Leaf Area Meter LI-3000 (Li-Cor, Inc., Lincoln, NE, USA).

Determination of dry weights.

Propagation lettuce was harvested 20d after the beginning of filter treatments. Shoot dry weights were obtained by weighing the plant material after drying at 75 °C until a constant mass was reached.

Leaf thickness.

Leaf thickness was measured at the central region of the lamina, adjacent to the mid-vein, using a 0-25mm micrometer (RS Components, Corby, UK).

Epidermal cell size and cell numbers.

At 18 d after emergence, leaf two of lettuce was removed following the cessation of growth, in order to measure epidermal cell size using the dental rubber impression technique (Weyers & Johansen 1985; Poole *et al*. 1996). Measurements were made at the central region of the lamina. The procedure involved first covering the leaf surface with dental impression material (Xantopren, Dental Linkline, UK) to make an imprint of the epidermal surface area. Once the material had set (30-60 s) the leaf was peeled away. Acrylic-based nail varnish was used to produce a translucent positive replica from the negative rubber impression. Cell size was measured at 400X magnification using a Leitz 'Labovert' (Leica, UK) microscope fitted with a ½ inch CCD digital video camera (JVC, Japan). Final leaf area of leaf two was also determined before harvest using the method described in a previous section.

Photosynthesis measurements.

Measurements of light saturated photosynthesis (1500 μmol m^{-2} s^{-1}) using a Portable infra-red gas analysis system (CIRAS- 1, PP systems, Hitchin, UK) were made on leaf two of lettuce, always between 9:00 a.m. and 13:00 p.m., starting six days after leaf emergence and continuing for 14 d.

Extraction and assay of cell wall peroxidase activity.

Starting immediately following emergence and for 6 subsequent days leaf two was harvested and fresh weights were determined for each sample. These were then stored in liquid nitrogen for subsequent assay of cell wall-associated peroxidase activity. Extraction of cell wall peroxidase was carried out using the method outlined by Bacon *et al*. (1997). Briefly, samples were homogenised in liquid nitrogen before being transferred to ice cold buffer (10mM sodium succinate, 10mM calcium chloride, 1mM dithiothreitol; Sigma, Poole, U.K.), pH 6.0, at a ratio of 10:1 buffer to sample fresh weight. 200 µl of buffer and homogenised tissue was then centrifuged at 2000g for five minutes. The pellet was washed four times in 200 µl of 10mM sodium succinate (pH 6.0) to remove cytoplasmic peroxidase activity, before being re-suspended in an equal volume of final extraction buffer (50mM sodium succinate, 1M sodium chloride, pH 6.0) to extract activity from the cell wall. The four low ionic concentration buffer wash stages reduced activity derived from tissue by c.95% (data not shown). The final high salt wash extract showed an increase in activity when compared with the final wash of the four low salt concentration washes. Activity was determined by assaying a 100 µl sample of the supernatant using the guaicol method detailed by Chance & Maehly (1955).

Field trials.

Commercial lettuce field trials were carried out beginning on the 29 May 2002 and continued over three UK growing seasons with three plantings in each season. In all instances plants were removed from their respective spectral filters and planted out in a random block design at Stockbridge Technology Centre, North Yorks, UK.

Statistical analysis.

Multiple Student t-tests were used in all analysis except when calculating daily leaf expansion in lettuce. Because the same leaves were measured throughout the lettuce growth experiment leaf area data was analysed using two way, repeated measures ANOVA with *post hoc* multiple pairwise comparison using Tukey tests to investigate the effect of treatments on leaf area during development. All analyses were performed using Sigmastat V 2.03 (SPSS Inc.).

Results

MEASUREMENT OF LETTUCE LEAF EXPANSION FOLLOWING LEAF 2 EMERGENCE

Following the beginning of filter treatments, which was approximately 6 days prior to beginning of measurements, there was no delay in leaf 2 emergence (Table 1). Following leaf 2 emergence, in Standard and UV-opaque, the expansion rate in leaf 2 increased logarithmically to peak 7 days after emergence, at which point lamina area was increasing by ~900 mm^2 day^{-1} (Fig. 1). Thereafter, expansion slowed progressively, with little (<10 mm day-1) taking place after 17 days (Fig. 1). The UV-transparent treatment caused significant decreases in the expansion rate of leaf 2 during the period of maximum expansion (two-way repeated measures ANOVA, treatment x time interaction, $F_{14,52} = 39.89$; $P<0.001$; Fig. 1). Daily leaf expansion rates were significantly reduced in UV-transparent compared to Standard on day 6 ($P<0.01$, according to Tukey tests for individual days; Fig 1) and days 6 and 7 when compared to both Standard and UV-opaque treatments ($P\leq0.01$; according to Tukey tests for individual days; Fig.1). In UV-transparent plants the maximum rate of leaf

expansion was reduced to approximately ~600 mm^2 day^{-1} which led to reduced final leaf area (Kruskal-Wallis one-way ANOVA, $P<0.05$; Fig. 1).

FINAL LEAF AREAS OVER 3 YEARS OF EXPERIMENTS

Over the twelve repeats of the propagation experiments between May 2003 and September 2006 the UV-transparent film consistently caused significant reductions in the final area of leaf two of Iceberg lettuce relative to both Standard and UV-opaque produced crops (Table 1). Averaged across all experiments, UV-transparent film significantly reduced ($P\leq0.05$) leaf area between 3% $\pm$ 19% and 19% $\pm$ 31 % compared to Standard and UV-opaque propagated crops respectively (Table 1). This reduction in total leaf 2 area in UV-transparent was predominantly a function of significantly reduced leaf length coupled with small, mostly non-significant, increases in leaf width (data not presented).

LEAF THICKNESS OVER 3 YEARS OF EXPERIMENTS

The thickness of leaf two was significantly increased in plants grown under the UV-transparent when compared to both Standard (20%, $P<0.001$) and UV-opaque (10%, $P<0.001$) filters (Table 2).

LEAF 2 DRY WEIGHT OVER 3 YEARS OF EXPERIMENTS

A similar pattern was observed in the dry weight data. The UV-transparent filter significantly increased dry weights relative to Standard (14%, $P<0.05$), although there was no significant effect on dry weights when compared to UV-opaque (7%, $P>0.05$, Table 2).

PHOTOSYNTHESIS

CO_2 assimilation rates (μmol m^{-2} s^{-1}) in leaf 2 of UV-transparent plants (11.99 ± 0.69) did not differ significantly from those in leaf 2 of Standard (11.25 ± 0.78; two-way repeated measures ANOVA, $F_{36,103}$ = 6.21, P>0.05; Fig. 2) or UV-opaque (10.17 ± 0.65; two-way repeated measures ANOVA, $F_{36,103}$ = 9.39, P>0.05; Fig. 2) over the 12-day experiment. There was no significant effect on assimilation rates between Standard and UV-opaque (two-way repeated measures ANOVA, $F_{36,103}$ = 2.21, P>0.05; Fig. 2).

EPIDERMAL CELL SIZE MEASUREMENTS

In a separate experiment, a destructive harvest of leaf 2 was carried out after 20 days. Final leaf area was reduced in UV-transparent plants when compared to UV-opaque (19%, P<0.001; Table 3) and while there was a 10% reduction compared to the Standard treatment this was not significant (P>0.05; Table 3). The only effects of treatment on epidermal cell numbers per leaf was a 19% increase in UV-opaque compared to UV-transparent (P<0.05; Table 3). UV-opaque also significantly increased epidermal cell area compared to UV-transparent (P<0.001) but not Standard (P>0.05) (Table 3). There was a significant reduction in final epidermal cell areas in UV-transparent relative to both the Standard treatment (9 %, P<0.05) and UV-opaque (17%, P<0.001) (Table 3).

CELL WALL PEROXIDASE ACTIVITY

Cell wall peroxidase activity remained relatively constant in leaf 2 of UV-opaque plants throughout the 6 day experiment (Fig. 3). Leaf 2 of Standard showed significant increases in activity throughout the six day experiment when compared to the UV-opaque treatment only (two-way ANOVA, $F_{36,47}$ = 111.47, P<0.001; Fig. 3).

Also, leaf 2 of the UV-transparent treatment exhibited increased levels of cell-wall associated peroxidase activity compared to both the Standard (two-way ANOVA, $F_{36,47} = 5.35$, $P<0.05$; Fig 3) and UV-opaque treatments (two-way ANOVA, $F_{36,47} = 5.35$, $P<0.001$; Fig 3) throughout the 6 day experiment. Peroxidase activity in leaf two of Standard and UV-transparent exhibited the greatest increase in activity relative to the UV-opaque treatment on day 3 ($P < 0.05$, Tukey tests for individual days; Fig. 3) and day 4 respectively ($P < 0.01$, Tukey tests for individual days; Fig. 3).

FIELD TRIALS FOR SPECTRAL FILTER PRODUCED ICEBERG LETTUCE OVER 3 YEARS OF EXPERIMENTS

Over the 3 repeats of the experiment where lettuce was grown to the point when they would be commercially harvested, the UV-transparent modifying film produced an significant increase in mean harvested crop fresh weight by 23% when compared to Standard ($P<0.001$) and by 9% non-significant increase relative to UV-opaque ($P>0.05$) (Fig. 4). The Standard filter produced the lowest fresh weights of all treatments reducing harvests by between 23%, ($P<0.001$) and (15%, $P<0.05$) compared to UV-transparent and UV-opaque respectively (Fig. 4).

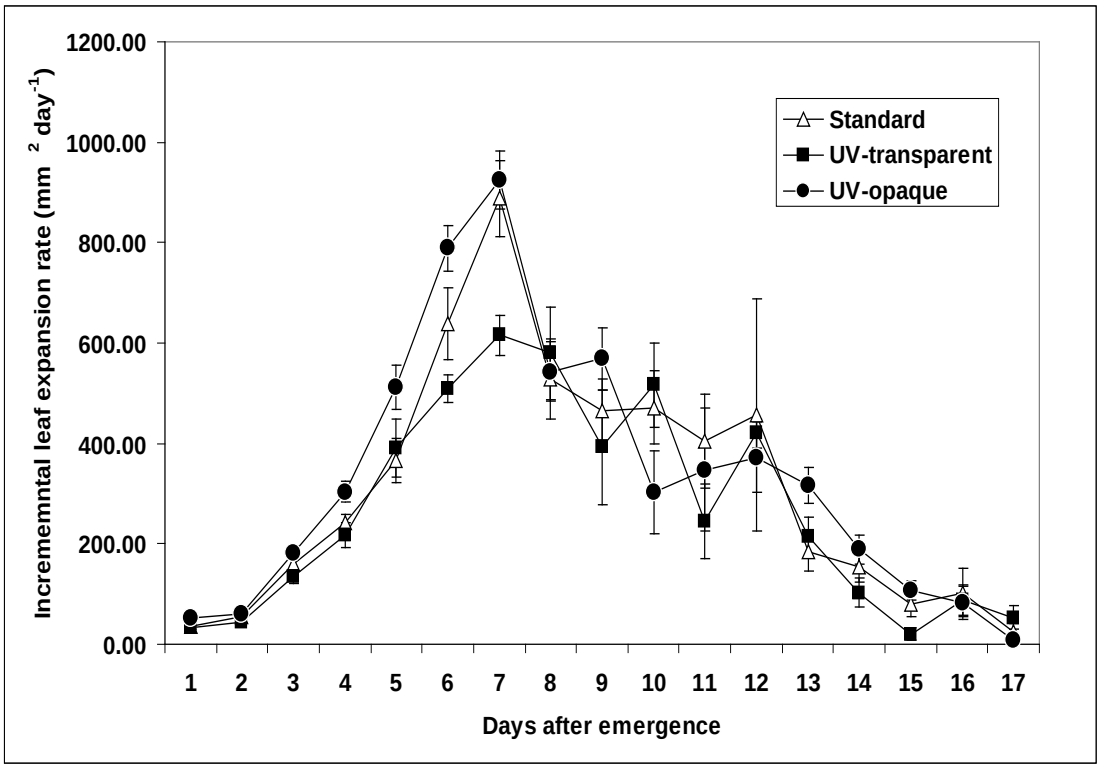


Figure 1. Effects of the treatments on the expansion of leaf 2 of propagation lettuce. Data presented as daily incremental leaf expansion ± S.E. for all treatments.

Table 1. Effect of filter treatments on days to emergence and final leaf area. Final leaf area measurements were taken at 20 days after treatment began. Each value is the mean ± S.E. of 15 replicates.

	Standard		UV-transparent		UV-opaque	
Parameter	Mean	S.E.	Mean	S.E.	Mean	S.E.
Days to emergence	5.29	0.16	5.07	0.25	5.49	0.41
Final leaf area (cm^2)	52.54	2.6	45.78	1.7	56.63	1.6

Table 2. Effect of filter treatments on final leaf thickness and final leaf 2 dry weight of propagation lettuce. Measurements taken at 20 days after leaf 2 emergence. Each value is the mean ± S.E. of 12 replicates for leaf thickness and ≥ 20 replicates for final dry weights.

	Standard		UV-transparent		UV-opaque	
Parameter	Mean	S.E.	Mean	S.E.	Mean	S.E.
Final leaf thickness (mm)	0.336	0.016	0.416	0.016	0.371	0.006
Final leaf 2 dry weight (g)	0.024	0.001	0.028	0.001	0.026	0.001

Table 3. Effect of filter treatments on final leaf area, final epidermal cell area and final cell numbers per leaf of propagation lettuce. Measurements taken at 20 days after treatment began. Each value is the mean ± S.E. of 12 replicates.

	Standard		UV-transparent		UV-opaque	
Parameter	Mean	S.E.	Mean	S.E.	Mean	S.E.
Final leaf area (cm^2)	167.39	7.87	149.98	7.77	217.05	7.45
Final cell area (μm^2)	209.12	12.33	189.28	10.14	228.90	13.57
Final cell number per leaf ($x10^4$)	84.69	7.34	80.46	3.17	99.66	6.78

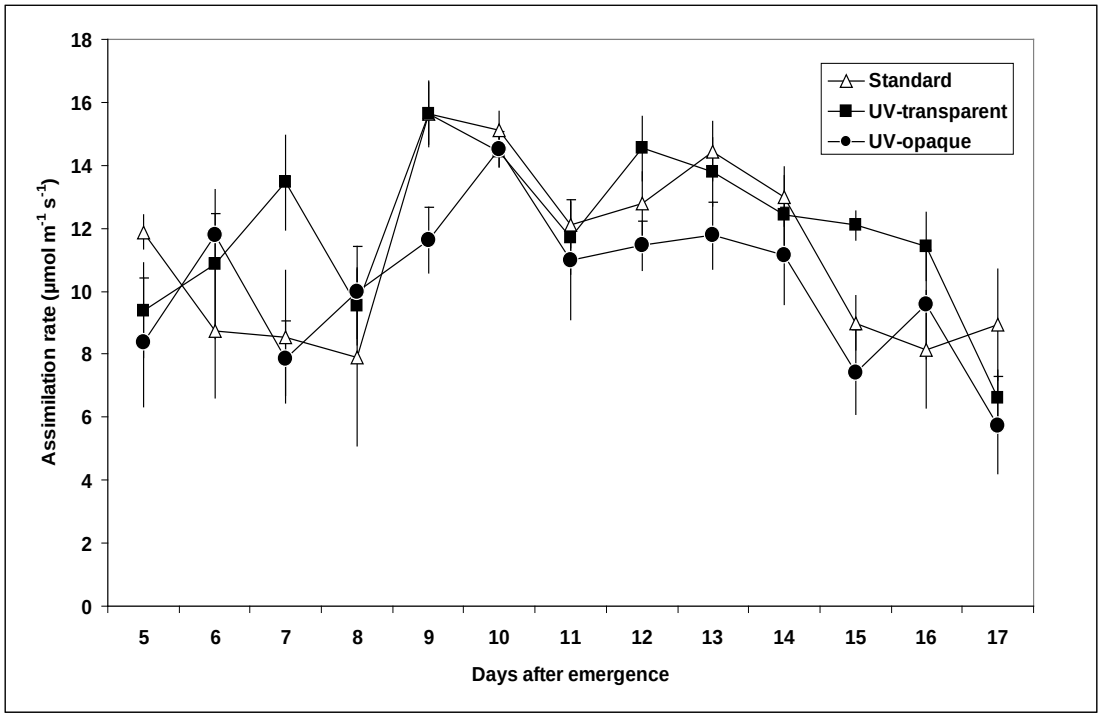


Figure 2. Assimilation rates expressed as µmol m^{-1} s^{-1} in leaf 2 of propagation lettuce in Standard (Δ), UV-transparent (▮) and UV-opaque (●) treatments between day 5 and day 17 following emergence (n=4).

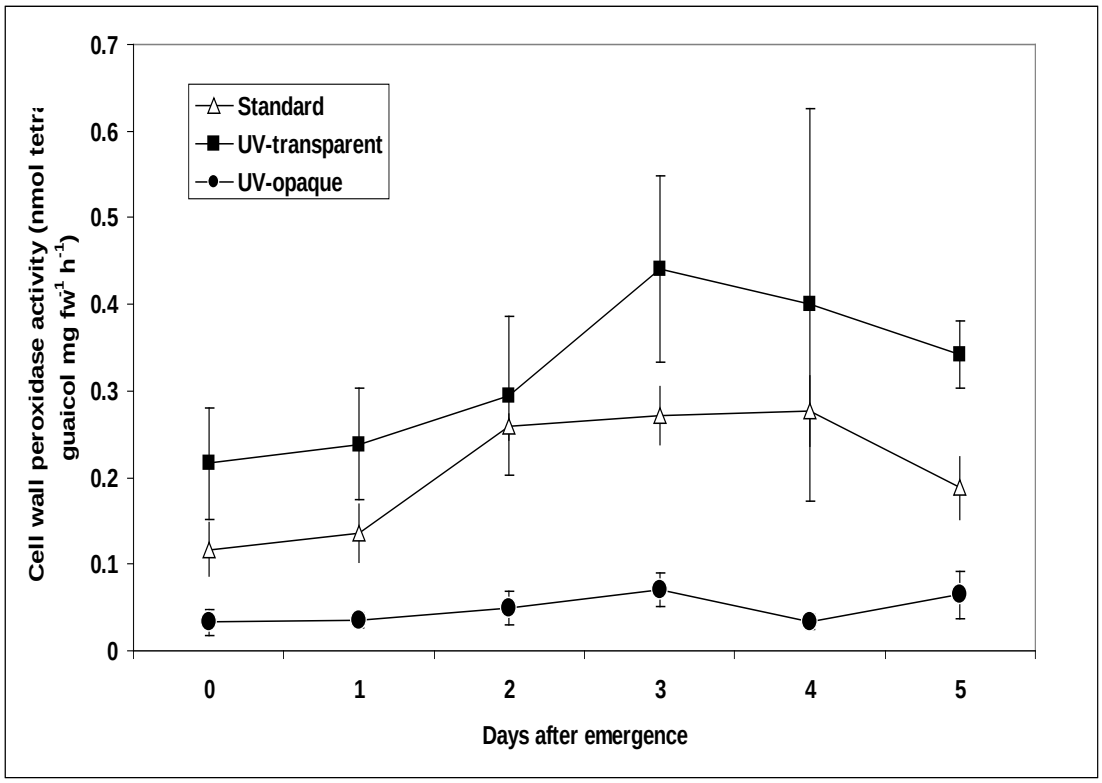


Figure 3. Cell wall-associated peroxidase activity (expressed per nmol tetraguaicol mg-1 FW h-1) in leaf 2 of propogation lettuce in Standard (Δ), UV-transparent (▮) and UV-opaque (●) for 6 days following treatments (n=4).

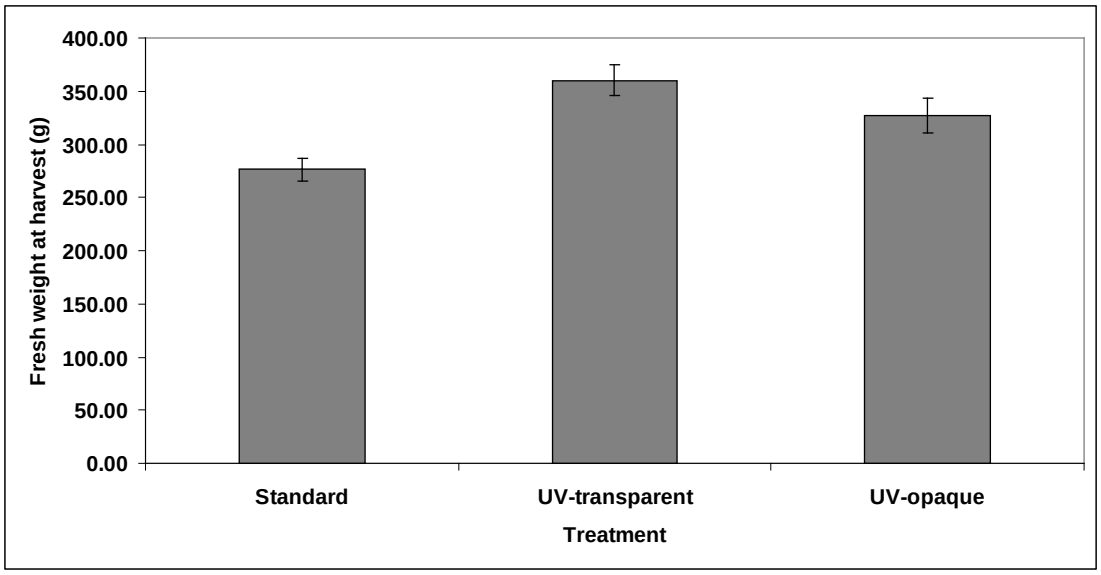


Figure 4. Effect of treatments on harvested yields in tunnel propagated lettuce . Measurements taken ≥ 92 days after the end of the propagation stage. Each value is the mean ± S.E. of ≥ 26 replicates.

Discussion

What is clear from this four year investigation in lettuce is that cladding film with high UV-transmission properties has the potential to deliver commercially useful growth regulation at the propagation stage. Limited exposure (14 days) to relative altered high solar UV produced crops with significantly reduced leaf expansion but increased leaf thickness compared to those crops produced under both the Standard and UV-opaque films (Fig. 1 & Tables 1-3). In lettuce the UV-transparent film was effective in producing a crop at the end of the propagation stage with morphological characteristics desired by growers; reduced leaf expansion coupled with increased leaf thickness and dry weight biomass. The rate of leaf expansion was increased along the length axis only under the Standard, and to an even greater degree, the UV-opaque filter with no corresponding increase in leaf thickness (data not presented and Table 2).

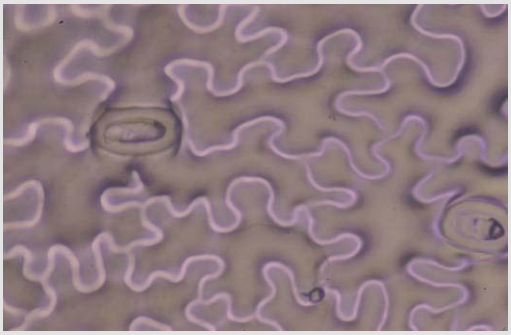
The capacity for UV modification within the ambient range to control growth is not surprising given the basic photobiological information on the exclusion of UV-B (20-28) and the more limited data on UV-A exclusion (20-22). That basic photobiological literature also suggests strongly that plant responses to UV manipulation would vary strongly between species or genotype. However, in this study lettuce, and in separate studies with cauliflower (see HDC, Project CP19 report 2007), both crops exhibited similar morphological adaptations to altered solar UV. Also, there is little evidence that the effects of UV manipulation are more variable over time in these crops. For example, the UV-transparent film significantly and consistently reduced leaf two expansion while producing an increase in leaf thickness over the six repeats and four years of the experiments with propagation lettuce.

The reduction in leaf expansion in UV-transparent is not a function of reduced carbon fixation. Indeed, the rate of photosynthesis in lettuce grown under this filter was marginally, but not significantly increased, when compared to the Standard and UV-opaque produced crops (Fig. 2). Therefore there was no link between carbon fixation and the reduction in leaf expansion at the propagation stage. The effect of the UV-transparent filter on final leaf area could largely be attributed to a reduction in epidermal cell area: there was no significant reduction in cell numbers when compared to Standard (5%) but a 19% reduction relative to the UV-opaque crop (Table. and graphic 1 below).

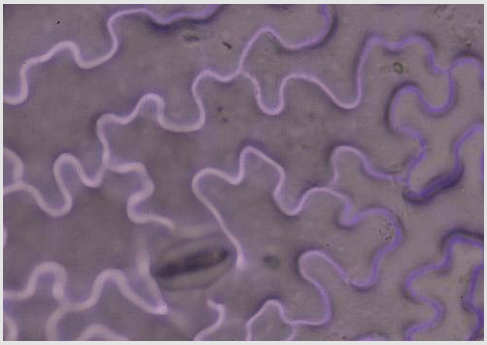
The regulation of leaf expansion through changes in cell size is complex (Fry 1986). As well as changes in turgor (there is no evidence of altered water relations in plants grown under the spectral filters), cell wall extensibility is regulated by several

enzymes including xyloglucan endotransglycosylase (XET; for review see Campbell & Braam 1999); expansin (Lee & Kende 2001); and cell wall peroxidases (Hohl, Greiner & Schopfer 1995).

The role of cell wall peroxidases in regulating growth processes (Penel et al. 1992) through the control of cell wall plasticity during cell elongation is well documented (Hoson, Wakabayashi and Masuda 1995), with plant growth hormones such as abscisic acid or methyl jasmonate related to an increase in its activity (Tse-Min & Yaw-Huei 1996). These enzymes can increase oxidative cell wall cross-linkages which fix the viscoelastically extended wall structure, leading to the regulation of tissue growth by conferring irreversibility to wall extension (Hohl et al. 1995). Stresses including pathogen (Scott-Craig et al. 1995) and herbivore (Moore et al. 2003a and 2003b) defence reactions and artificial wounding (Angelini, Manes & Federico 1990; Kawaoka et al. 1994) can change peroxidase activity but as far as we are aware there has been no previous link made between crop exposure to differing solar UV levels and changes in cell-wall associated peroxidise activity.



Leaf 2 epidermal cells in UV-transparent



Leaf 2 epidermal cells in UV-opaque

We show here that cell wall peroxidase activity is upregulated in response to exposure to increased solar radiation (Fig. 3). The increase in peroxidase activity reported here was associated with a reduction in the growth rate of leaf 2 on day 7 (Fig. 1). The timing of the increase in peroxidase activity immediately following leaf emergence also appears consistent with non-significant reductions in leaf expansion under UV-transparent at day 4; becoming significant on day 7 (Fig. 1). Our data are consistent with a causal role for solar UV-induced upregulation of cell wall peroxidase in the inhibition of leaf expansion in propagation lettuce. The same mechanism may also partly account for the increased crop performance in commercial field trials through changes in crop leaf tissue mechanical strength and / or changes in plant biochemistry linked to resistance to the multiple abiotic and biotic stresses.

In the case of propagation lettuce ‘short-stocky’ plants with both maximum leaf thickness and leaf tissue mechanical strength is required commercially to minimise plant damage during handling and transplanting in to the field; it is these characteristics that are thought to contribute to good crop performance in the field. Results from our field experiments, made over a period of three UK growing seasons, provide strong evidence that propagation under crop covers with broad high solar UV transmission produce crops that deliver significantly greater final yield (Figs. 4.a, 5.b. & 6.b).

In conclusion, exposing propagation crops to maximum solar ambient UV while maintaining protection from wider environmental stresses under protective structures induce beneficial morphological changes in a variety of UK important crops. Our data indicates an important role for peroxidise mediated increases in leaf thickness as a

component of improved long-term crop performance in the field but other known effects of UV on physiology or biochemistry may be equally important to final yield. Increasing, or even maintaining, crop quality while employing the least-cost input form of protective structure is a major priority for UK growers as pressure mounts on producers cut costs and the results of this study show that manipulation of the light environment in low-cost tunnel structures can contribute to this objective.

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PART 3: Can transgenerational resistance to insect pests be exploited in commercial crop production?

In response to attack by insect pests plants induce a wide range of defences for the purpose of minimising damage at the time of attack and reducing the likelihood of future attack. Because seeds of attacked plants develop in the maternal environment and that environment may predict the type of conditions that offspring will encounter, herbivory may have a delayed effect on plant fitness by acting to induce defences across generations of plants if the progeny of attacked plants are more resistant to attack than the offspring of undamaged plants. We report here that Tomato (*Lycopersicon esculentum* Mill cv. Carousel) repeatedly treated with Jasmonic acid (JA), a known chemical elicitor of induced resistance to herbivores, every three days from emergence of the first true leaf to harvest of the first ripe fruit increased resistance to *Tetranychus urticae Koch* in the first generation progeny by 56% and 12% in two separate experiments carried out over two UK growing seasons compared to controls. In these same experiments the number of *Tetranychus urticae* offspring produced by each live adult at the end of the experiment was reduced in treated progeny by 35% and 37% respectively. There was no effect of jasmonic acid treatment in the maternal generation on seed mass, time to emergence or vegetative biomass in progeny. The results from both these experiments demonstrate that the maternal environment determines, at least in part, the defensive phenotype of progeny. We discuss the underlying epigenetically inherited mechanism mediating such transgenerational responses and how this effect could be exploited through simple changes to current commercial seed production practices for the purpose of cost effectively reducing the damage caused by economic pests and therefore the requirement for pesticides in commercial crop production.

Introduction

Most plants are subject to parasitism by chewing insects and the associated damage can negatively impact plant fitness (Agrawal 2000; Moore et al 2003). For this reason herbivory may select for defences that act to reduce the frequency and scale of attack, or to reduce the growth and / or reproductive consequences for the plant (Agrawal 2002). Because plants as sessile organisms and have no way of avoiding injury caused by chewing insects or large herbivores they have evolved pre-existing physical barriers that act to minimise damage. These include the cuticle, which restricts herbivore grazing, or trichomes and thorns, which make access to certain plant parts

difficult (Gomez & Zamora 2002; Karban & Baldwin 1997). However, if these barriers fail and the plant is injured, cells are capable of mounting a defence response through the transcriptional activation of specific genes (Leon, Rojo & Sanchez-Serrano 2001). The initiation of these responses act to direct the healing of damaged tissue and stimulates defence mechanisms for the purpose of minimising future damage (Leon, Rojo & Sanchez-Serrano 2001).

Depending on the species, and the type and level of damage caused, local defence responses may be activated within minutes, or perhaps hours, and include the generation, perception and transduction of signals leading to defence gene activation (for review see de Bruxelles & Roberts 2001). The proteins these genes encode act to inhibit herbivore performance by changing the digestibility of the tissue (Jongsma *et al*. 1995), or through toxin synthesis (Griffitts *et al*. 2001), but also mediate wound repair (Leon, Rojo & Sanchez-Serrano 2001), and play a role in altering plant metabolism (Broddmann *et al*. 2002). Such inductive responses can be adaptive in that their activation can increase the fitness of plants in the presence of herbivores (Agrawal 1998).

While herbivory leads to the activation of defence related changes it always by necessity in nature, where resources are often limited, leads to a reduction in leaf area and resources for the plant. Such reductions in the ability of the plant to fix resources can directly decrease growth, survivorship and reproductive success (Karban 1997). Therefore herbviory can directly influence plant fitness by affecting the number and / or vigour of the plants progeny. However, in a commercial cropping environment resources are far from limited and therefore we propose here that herbivory may have

a delayed effect on plant fitness by changing the behaviour of its progeny to insect pests. Seeds develop in the maternal environment and that environment may predict the type of conditions that offspring will encounter. Thus such transgenerational effects of herbivory may provide, through a so-far unidentified mechanism, an instrument for adaptive maternal changes in plant induced resistance.

In separate experiments carried out during two UK growing seasons we carried out an investigation of such effects in a commercially grown tomato crop. We hypothesised that repeated activation of the pathways linked to defence against herbivores using a recognised chemical inducer of resistance, jasmonic acid, from germination through to fruit production would produce more resistant progeny. We discuss the possible underlying epigenetically inherited mechanism mediating such transgenerational responses and how this effect could be exploited, through simple changes to current commercial seed production practices, for the purpose of reducing the damage caused by economic pests and the requirement for pesticides in crop production.

Materials & methods

PLANT MATERIAL AND JASMONIC ACID TREATMENT

Seeds of Tomato (*Lycopersicon esculentum* Mill cv. Carousel) were sown in Levington no. 2 compost (Keith Singleton, Egremont, UK) in 58 cm^3 commercial blocks to germinate. At 35 days they were transferred individually into 160 mm plastic pots filled with Levington no. 2 compost before being split into two completely random groups of 15 plants each and were isolated (so pollination only occurred within treatment populations using hand pollination) in individual glasshouses at Stockbridge Technology Centre, North Yorks, at a temperature (26ºC

day/ 13ºC night ± 3.0ºC) in natural light during June-July 2005 and the second experiment was carried out during the same period in 2006. Leaves only of the Jasmonic Acid (Sigma, UK) treatment group were sprayed to dripping point with a solution of 3mM JA in 0.2% ethanol every 3 days until the first fruit had ripened and was then harvested. Controls were similarly treated with 0.2% ethanol only and the first fruit was similarly harvested. One seed was randomly chosen from each of the control and JA treated fruit.

For the first experiment these were weighed immediately and then germinated in isolated glasshouses as described above at a temperature (18ºC day/ 10ºC night ± 4.0ºC) in natural and supplemented light during November 2005 - March 2006. For the second experiment the same procedure was used but seed were dried and stored for 248 days before being germinated as detailed above. First generation progeny plants from both maternal Control and JA treatments were allowed to develop under identical conditions, with no treatment, until full expansion of the fourth leaf at (95d for experiment one) and (106 days for experiment two) post germination at which point *Tetranychus urticae* were applied.

APLICATION OF *TETRANYCHUS URTICAE*

Six adult female (2-3 days old) *T. urticae* were placed on the middle leaflet of the first fully expanded leaf of a tomato plant (*L. esculentum* Mill cv. Carousel). There were 10 plants per treatment. Each plant was grown in a 3L pot in a glasshouse (16L: 8D, min^{m} temp. 15^{o}C, venting at 24 ^{o}C). After seven days the plants were removed and the numbers of live adult and offspring were recorded for each treatment.

RESULTS

Experiment 1

There was no effect of maternal treatments on seed weight (P>0.05, Kruskal-Wallis one way ANOVA; data not presented) or time to emergence in first generation JA treatments (11 $\pm$ 2.96) when compared to Controls (10 $\pm$ 1.47) (P>0.05, Kruskal-Wallis one way ANOVA; data not presented).

Repeated application of JA to maternal plants resulted in a 41% reduction in the number of live adults after 7 days on first generation progeny when compared to first generation Controls (P<0.05, Students t-test, Fig 1.a). The total number of eggs produced was also reduced by 56% in JA treatments (P<0.001, Students t –test; Fig. 2.a) and although there was a 35% reduction in the total number of eggs produced per live adult at the end of 7 days this did not represent a significant reduction (P>0.05, Students t-test, Fig. 1.c).

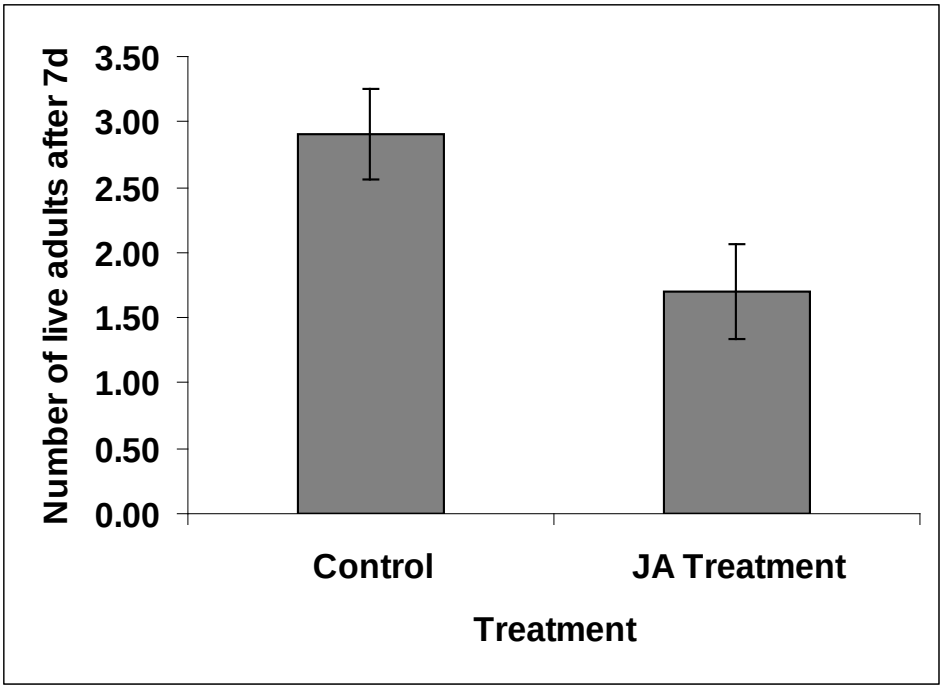
Experiment 2

Similar to results obtained in experiment one there was no effect of maternal treatments on seed weight (P>0.05, Kruskal-Wallis one way ANOVA; data not presented) or time to emergence in first generation JA treatments (9 $\pm$ 1.19) when compared to Controls (8 $\pm$ 3.6) (P>0.05, Kruskal-Wallis one way ANOVA; data not presented).

Again, in this second experiment repeated application of JA to maternal plants and, in this case, dry storage of seed for 8 months resulted in 12% reduction in the number of

live adults after 7 days on first generation progeny relative to first generation Controls (Students t-test, Fig 1.b). In this experiment the total number of eggs produced was reduced by 51% in JA in first generation JA treatment progeny ($P<0.001$, Students t-test; Fig. 2.b) and a 37% reduction in the total number of eggs produced per live adult at the end of 7 days ($P>0.05$, Students t-test, Fig. 3.b).

a)



b)

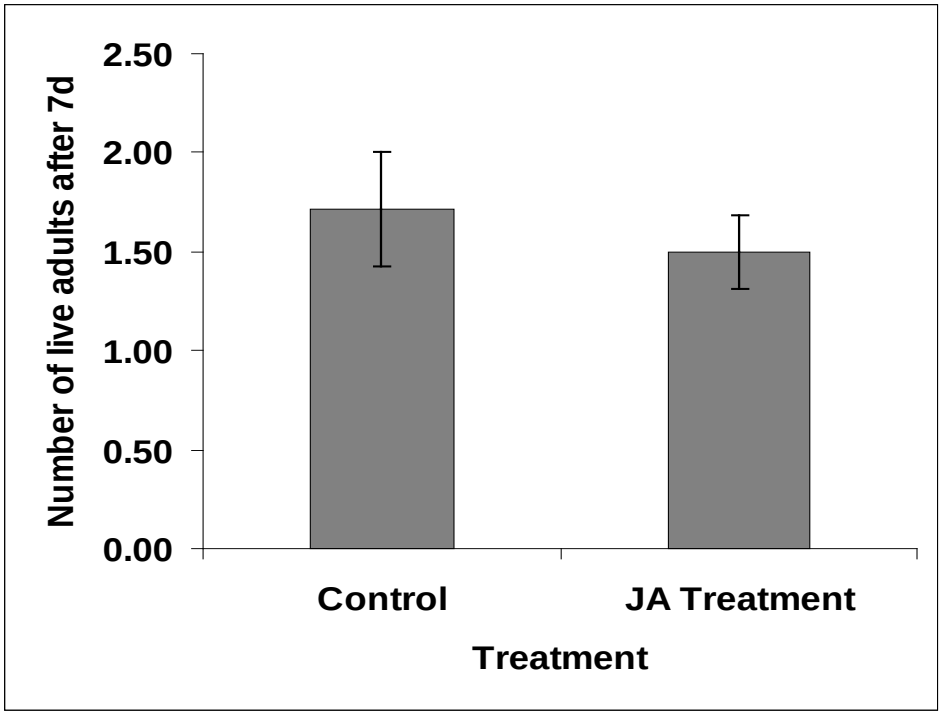
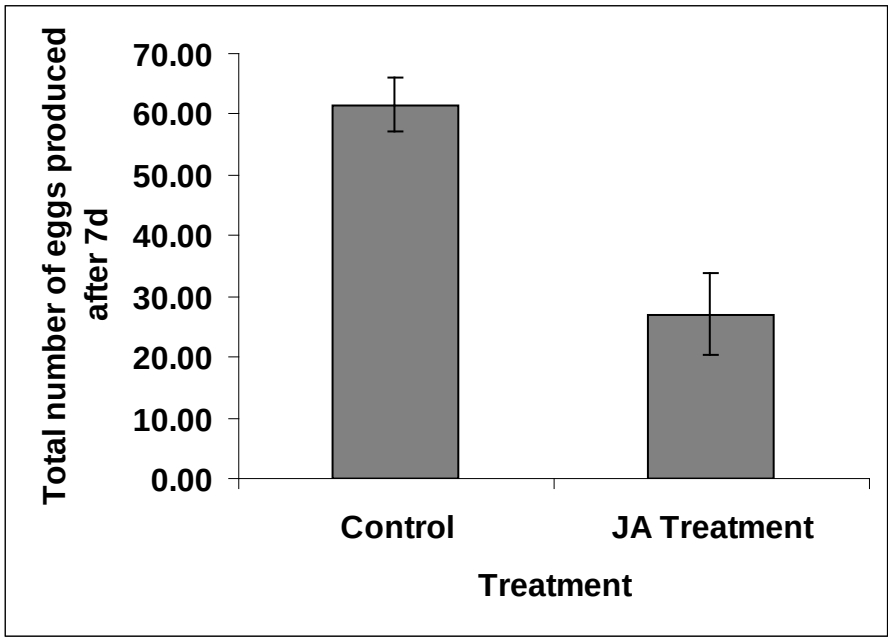


Figure 1. Number of live adult mites on (*Tetranychus urticae)* per unit area of leaf four on first generation progeny plants of JA treated and Control tomato cv. Carousel 7 days after challenge in a) summer 2005 experiment and b) summer 2006 repeat experiment. n=10 + s.e.

a)



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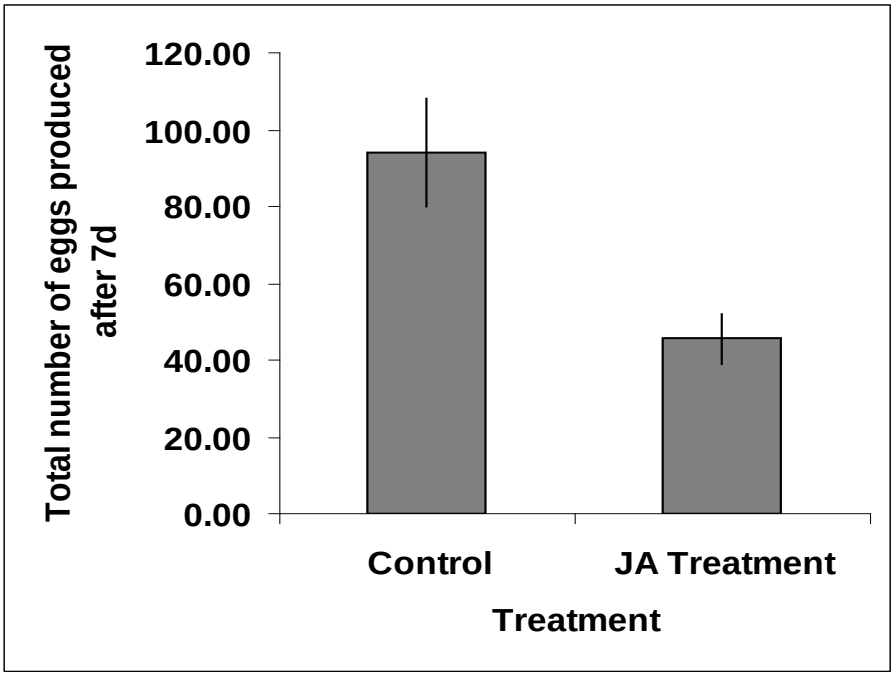
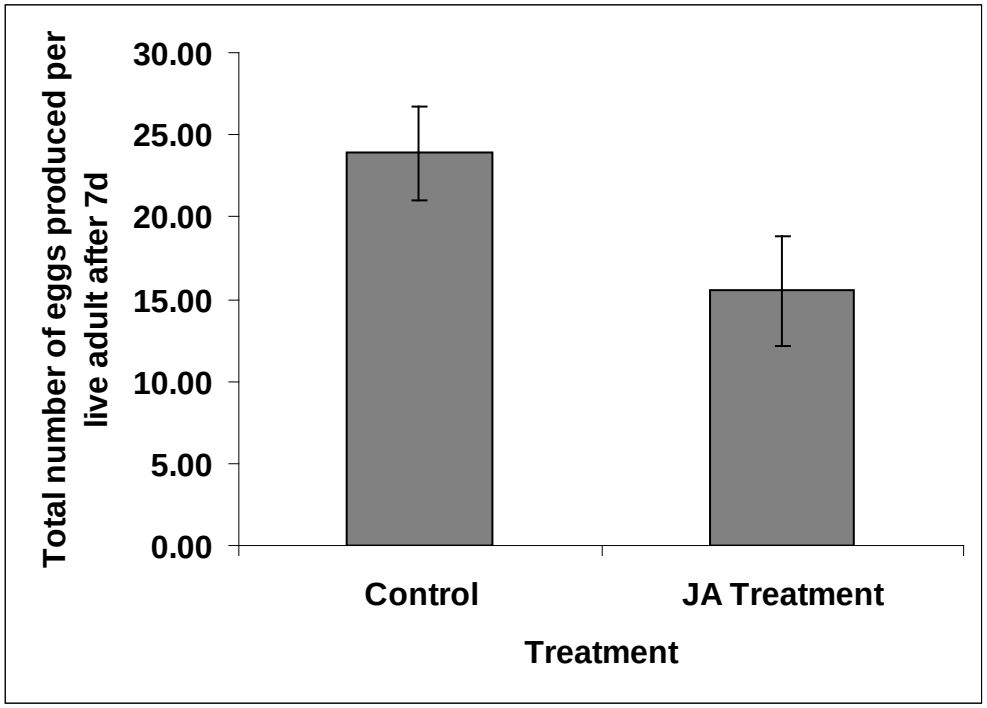


Figure 2. Total number of eggs produced by *Tetranychus urticae* on first generation progeny plants of JA treated and Control tomato cv. Carousel 7 days after challenge in a) summer 2005 experiment and b) summer 2007 repeat experiment. n =10 + s.e.

a)



b)

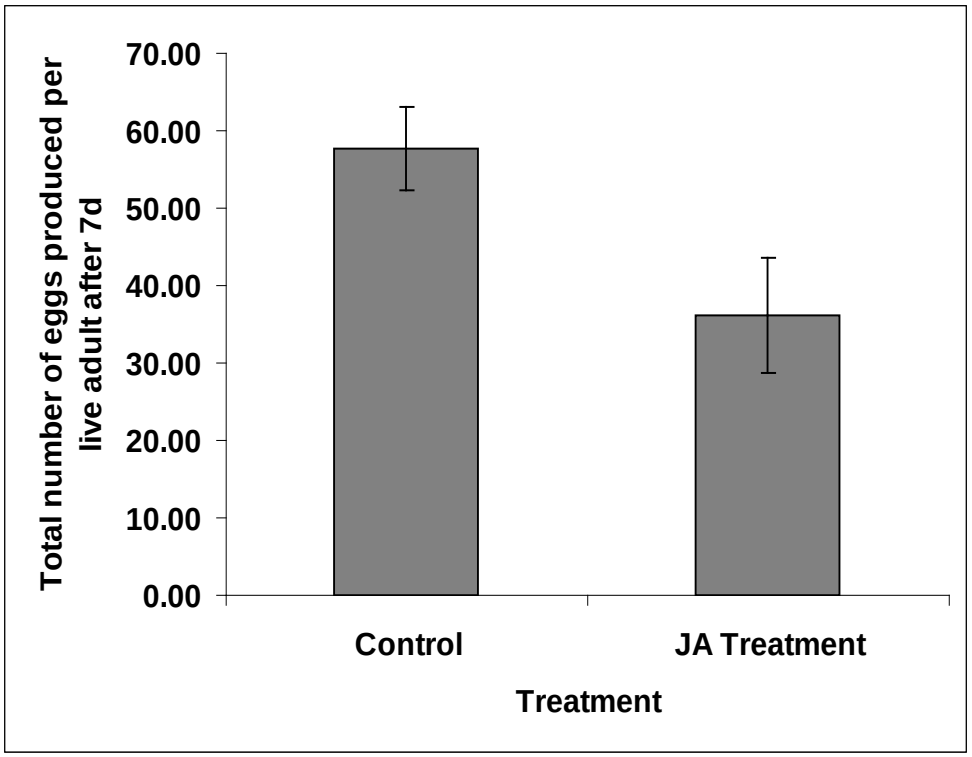


Figure 3. Total number of eggs produced per live *Tetranychus urticae* on first generation progeny plants of JA treated and Control tomato cv. Carousel 7 days after challenge in a) summer 2005 experiment and b) summer 2007 repeat experiment. n=10 + s.e.

DISCUSSION AND FUTURE WORK

The repeated induction of resistance in maternal plants had an effect on the resistance of first generation progeny to *Tetranychus urticae* (Figs. 1.b. – 2.b). Repeated treatment of maternal plants with jasmonic acid (JA) decreased *Tetranychus urticae* survival by 41 and 12% and the number of eggs produced by 35 and 37% compared to controls. There was no effect of treatments on progeny seed mass (Figs. 1.a) or the time to emergence (data not presented).

The results of these two separate experiments provide strong evidence that repeated priming of the pathway(s) linked to herbivore resistance in *Lycopersicon esculentum*, using exogenously applied JA, causes maternally induced resistance in porgeny. In this study maternal plants were treated with JA from emergence through to the time when the first fruit had ripened, although fruits were isolated at the time of spraying and only foliar tissue was targeted. Thus the maternally inducing signal must be translocated from the vegetative tissue, through the developing fruit to the seed. In order to explain the increased levels of first generation progeny resistance reported here this signal must then act to alter the behaviour of the embryo through development into early maturity (>95 days) as a first generation hereditary trait.

For three decades, largely due to the work of Richard Dawkins, it has been widely accepted that the fundamental unit of selection is the gene, the unit of heredity (Dixon & Dawkins 1988). This hypothesis has so dominated discussion that other levels of selection or forms of inheritance has been largely ignored. However, recent studies from both the animal and plant kingdoms suggest that perhaps too much emphasis has

been put on the gene-centred approach and that there are forces acting on evolution other than orthodox genetics (Jablonka & Lamb 1998; Regev et al 1998). There is now growing evidence that non-genetic information affecting development is routinely passed from one generation to the next (Jablonka 2003). This developing paradigm suggests that certain chemical ‘methyl’ groups “hitchhike” on genes leading to different interpretations of that gene. This heritable, non-genetic “hitchhiking” is known as epigenetic inheritance. There are many examples of heritable changes transmitted by something other than genes and in a landmark study Anway et al (2005) reported that initial exposure to two pesticides reduced sperm counts in at least the subsequent four generations of male rats. The effect did not seem to be the result of changes in the DNA sequence, making it the first time any chemical has been shown to cause an heritable effect other than by random mutation (Anway et al 2005).

There is also increasing evidence also from plant (Katop et al 2004; Agrawal 2002), bird (Naguib et al 2005) and insect (Mondor et al 2005; Podjasek 2005) studies that transgenerational effects act through epigenetically inherited changes in gene expression interpretation to influence offspring behaviour. In humans also the transgenerational effects of maternal nutrition or other environmental 'exposures' in human populations are becoming recognised (Khan et al 2005) and, perhaps surprisingly, recent work has indicated the possibility that exposure to certain compounds in men influences development and health in the next generation male (Pembrey et al 2005). They concluded that sex-specific, male-line transgenerational responses exist in humans and hypothesise that these transmissions are mediated by the sex chromosomes, X and Y.

Possible mechanisms of transgenerational resistance in plants

In plants, there is now initial evidence that newly acquired epigenetic states of transcriptional gene activity are transmitted to progeny (Takeda & Paszkowski 2006). This transgenerational inheritance of new epigenetic traits seems to rely on cytosine methylation maintained through meiosis and postmeiotic mitoses, giving rise to gametophytes (Takeda & Paszkowski 2006). DNA methylation in eukaryotic cells involves the addition of a methyl group to the carbon at position 5 of the cytosine ring. This reaction is catalyzed by the enzyme DNA methyltransferase (DNA-MTase) and this methylation reaction is the most common covalent modification occurring in eukaryotic DNA (Takeda & Paszkowski 2006).

Whether, and at what level, methylation acts to produce transgenerational changes in insect resistance in higher plants is not clear but one possibility is that it alters the synthesis of and / or the sensitivity to key molecules that regulate defence, possibly through increased production of receptors to those molecules. One such key signalling molecule linked to plant responses to pest resistance in all higher plants is jasmonic acid. JA is synthesised through the octadecanoid pathway and is a key regulator in the physiology, development and defence of plants with the complexity of this signalling pathway only just emerging (Schaller 2001; Schenk *et al*. 2000). Accumulation of JA is observed at high levels in damaged tissue and increases are also observed in systemic leaves making JA a leading candidate as the primary component of systemic, whole-plant responses to herbivory (Rojo *et al*. 1999; Laudert & Weiler, 1998). In this study repeated exogenous application of synthetic JA was applied to the maternal treatment group and this positively altered the behaviour of first generational

offspring to insect attack. If correct and methylation driven transgenerational changes in key defence related signalling pathways is utilised by plants for the purpose of increasing phenotypic plasticity in response to insect pest related stress it opens up the possibility to alter progeny behaviour to other important economic biotic and abiotic stresses.

Future possibilities

The body of evidence for transgenerational changes in plant behaviour through epigenetic inheritance is accumulating as it becomes increasingly evident that the gene-centred approach does not possess the explanatory power to account for the variety and complexity of organisms. Because epigenetic inheritance would appear to act very quickly, within the first generation offspring and in response to limited stress signalling (results reported here and Agrawal 2002) and in response to exposure to certain biologically active compounds (Anway et al 2005) this opens up the possibility of positively altering plant responses to biotic stresses in a straightforward and both economically and environmentally beneficial manner.

It is widely accepted that commercially produced crops are more susceptible to insect pests than wild populations and results reported here raise the possibility that this could be linked to the lack of biotic stress in the highly protected growing conditions of plants used for seed production. In a conventional system maternal plants are protected from pests using conventional pesticides in the belief that favourable growing conditions will produce the most productive progeny in terms of harvestable yield. However, if maternally induced resistance to insect pests is a wide-spread

phenomenon throughout the plant kingdom and plants grown for seed are protected from pests, then seed being produced for commercial crop production may be particularly susceptible. This susceptibility could be overcome by changing commercial seed production practice in economically neutral ways through the substitution of traditional pesticides for known chemical inducers of both pest and disease resistance. This approach should induce high levels of pest resistance in maternal plants as these compounds would act directly to upregulate defence mechanisms in maternal tissues meaning that leaf and therefore resource driven yield loss would be minimised in the first instance and in the second, positive epigenetically driven defence changes would be expressed in progeny. In conclusion, as well as providing an entirely new dimension to the study of gene–environment interactions, such transgenerational epigenetic driven effects in crop plants may provide a tool for altering crop behaviour in response to economically important biotic stresses without the requirement for genetic modification through minimal changes in commercial seed production techniques.

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PART 4: Potentiation of pathogen specific resistance in mature tomato by seed treatment with β-aminobutyric acid (BABA)

Summary

The non-protein amino acids γ-aminobutyric acid (GABA) and β-aminobutyric acid (BABA) have known biological effects in animals and plants. The mechanism by which they act *in vivo* has been the object of thorough research in animals and while it remains unclear in plants there is mounting evidence that BABA when applied as a foliar spray or root drench protects against a range of economically important pathogens. The role of jasmonic acid (JA) is much more well defined in terms of its ability to potentiate plant defence pathways linked to herbivore resistance when applied as a foliar spray. In a recent novel advance work carried out by our laboratory has shown that JA, when applied as a seed treatment, produces similar long-term resistance to a range of insect pests in a wide array of the worlds protected and arable crops as produced by a foliar spray. The purpose of this study was twofold: to determine if BABA would likewise induce disease resistance when applied as a seed treatment and investigate how JA singularly and as dual seed treatment with BABA would impact on resistance to *Oidium neolycopersici* in tomato. JA alone did not induce resistance to *O. neolycopersici*. Treatment with BABA significantly reduced disease severity at 20 and 35 days post-inoculation and the addition of JA to the seed treatment did not negatively affect BABA ability to mediate this observed resistance. Thus, a short 24-hour seed treatment with BABA provides a commercially relevant level of resistance to *O. neolycopersici* in mature tomato and simultaneous treatment with JA does not antagonise the pathways regulating this resistance.

Background

Plants must defend against a multitude of biotic agents across all spatial scales from pathogens that attack single cells to herbivores that remove large areas of a plants' photosynthesising material. This has led to the development of a myriad of constitutive defensive mechanisms for the purpose of reducing the level of damage at the time of attack and to reduce the likelihood and scale of future attack (Heil & Baldwin 2002). Apart from these constitutive defences herbivores and pathogens also induce at the time of attack several well-characterised plant defence and wound

response pathways (Nurnberger & Scheel 2001; Bostock 1999; Reymond & Farmer 1998; Enyedi *et al*. 1992). The types of induced defences activated depend on the nature of the threat. Following pathogen infection plant responses can be highly localised, possibly in the form of cellular death, or the so called hypersensitive response (HR) that restricts pathogen spread (Ger *et al*. 2002; Van Loon 1997). In contrast most, but not all, responses to free feeding herbivores act on a larger scale, given that herbivore attack usually creates damage over a greater area than localised pathogen attack (Walling 2000).

The type of biotic threat encountered will determine which signal transduction pathway is activated ensuring an appropriate spatial and temporal defence response (Ryals 1996; Genoud & Metraux 1999). In order for this to occur plants must have the ability to first identify, then prioritise each signalling pathway in such a way as to minimise damage from the current threat and reduce the likelihood of future damage, while preserving growth of both vegetative and reproductive tissues (Karban & Baldwin 1997).

In response to herbivory grazing plants have evolved pre-existing physical barriers that act to minimise damage, such as the cuticle, which restricts herbivore grazing, or trichomes and thorns, which make access to certain plant parts difficult (Gomez & Zamora 2002; Karban & Baldwin 1997). However, if these barriers fail and the plant is injured, cells are capable of mounting a defence response through the transcriptional activation of specific genes (Leon, Rojo & Sanchez-Serrano 2001). The initiation of these responses act to direct the healing of damaged tissue and stimulate defence mechanisms for the purpose of minimising future damage (Leon,

Rojo & Sanchez-Serrano 2001). Depending on the species, and the type and level of damage caused, local defence responses may be activated within minutes, or perhaps hours, and include the generation, perception and transduction of signals leading to defence gene activation (for review see de Bruxelles & Roberts 2001). The proteins these genes encode act to inhibit herbivore performance by changing the digestibility of the tissue (Jongsma *et al*. 1995), or through toxin synthesis (Griffitts *et al*. 2001), but also mediate wound repair (Leon, Rojo & Sanchez-Serrano 2001), and play a role in altering plant metabolism (Broddmann *et al*. 2002).

Even local limited damage can potentially activate any number of the aforementioned defence mechanisms in distal non-damaged parts of the plant (systemic response) as well as at the site of damage (local response) through the production of defence related signalling molecules (Guan & Scandalios 2000; Moyen & Johannes 1996 & Moyen *et al*. 1998; Cheong *et al*. 2002; Li, Schuler & Berenbaum 2002b; Stotz *et al*. 2002; Stotz *et al*. 2000). One such signalling molecule, jasmonic acid (JA) is known to act as a key regulator in the physiology, development and defence of plants and the complexity of this signalling pathway is only just emerging (Schenk *et al*. 2000). The biosynthesis of JA occurs through the octadecanoid pathway (Schaller 2001). The induction of jasmonates following attack by insects or pathogens are known to lead to the expression of defence mechanisms (Arimura *et al*. 2000; Wasternack & Parthier 1997) and plants with mutations that affect jasmonate synthesis are more susceptible to insect damage (Howe *et al*. 1996, McConn *et al*. 1997). In the majority of studies JA has been shown to up-regulate the synthesis of defence related metabolites (Reymond 2001; Wasternack *et al*. 1997), the accumulation of defence compounds, which includes many well characterised proteins (Tscharntke *et al*. 2001; Thaler *et al*.

1996; Thomma *et al*. 1998), the activation of defence genes (Moran & Thompson 2001; McCloud & Baldwin 1997) and toxic allelochemicals (Keinanen, Oldham & Baldwin 2001; Tscharntke *et al*. 2001).

Similarly when faced with attack by pathogens plants possess both constitutive and induced defences that help protect against pathogen damage (Heath 2000). Constitutive defences include chemical defences that are pre-formed, and possibly certain morphological characteristics, such as cuticle thickness (Manandhar, Hartman & Wang 1995) and the properties and density of the stomata in conjunction with the pre-existing composition and thickness of the epidermal cell wall (Kim *et al*. 2002). Chemical defences that are permanently active include certain phenolics (Franceschi, Krekling & Christiansen 2000), tannins (Machado *et al*. 2002) and sulphur containing compounds (Reichelt *et al*. 2002), which along with other types of plant molecules are known to confer a selective advantage against microbial attack (see Dixon 2001).

Plants are also able to erect defences at the time of attack, but in order for these induced defences to be successful there must be rapid recognition of the attacking pathogen (Rahman *et al*. 2002). This occurs through the recognition of specific pathogen (exogenous elicitors) or plant cell wall (endogenous elicitors) derived signalling molecules leading to defence gene activation (Ji, Smith-Backer & Keen 1998). One of the primary responses these genes code for is the hypersensitive response (HR) which is characterised by rapid, local death of plant cells at the sites of pathogen infection and is a common feature of non-compatible plant-pathogen interactions (Kumudini, Vasanthi & Shetty 2001). This programmed cell death (PCD) of affected tissue restricts the spread of pathogens from the infection site and is

considered one of the most effective plant resistance mechanisms, since it is highly effective in limiting pathogen spread (Jones 2001; Lam, Kato & Lawton 2001). Associated with HR is the activation of a diverse group of genes specifically geared towards defence (for details see: Glazebrook 1999; Silva *et al*. 2002; Dong, Chen & Chen 2003a; Gold & Robb 2002; Salles *et al*. 2002; Goldwasser *et al*. 1999; Zareie, Melanson & Murphy 2002 and Nishizawa *et al*. 2003).

The hypersensitive response is accompanied in many instances by the generation of locally synthesised signalling molecules including reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Bolwell 1999; Bolwell & Wojtaszek 1997), which occurs before and during lesion-associated host cell death (de Pinto, Tommasi & de Gara 2002; Pellinen *et al*. 2002) and within hours leads to the development of systemic acquired resistance (Morris *et al*. 1998). Systemic acquired resistance (SAR) is the fundamental defence mechanism induced by a wide range of pathogens (Cordelier *et al*. 2003; He, Hsiang & Wolyn 2002; Hennin, Diederichsen & Hofte 2002; Ryals *et al*. 1996). One response comprises a particular signal transduction pathway that begins as a biochemically synthesised response to the development of localised cellular necrosis, either as a function of (HR) (Dong *et al*. 2003), or alternatively, as a symptom of disease (Hammerschmidt 1999). Within hours of localised necrosis plants express (*PR*) genes at the site of infection and also systemically throughout the rest of the plant (Zareie, Melanson & Murphy 2002; Cordelier *et al*. 2003). The induction of SAR is thought to be dependent on the local production at the site of pathogen ingression and systemic diffusion to distal plant tissues of salicylic acid (Song & Goodman 2002; Siegrist, Orober & Buchenauer 2000; Rasmussen, Hammerscmidt & Zook 1991) leading to the expression of genes

and the production of numerous defence related proteins in crop species such as maize (Morris *et al*. 1998), tobacco (Song & Goodman 2002), soybean (He *et al*. 2001), and pepper (Lee, Kim & Hwang 2002).

Both the aforementioned induced resistance (IR) to herbivory and systemic acquired resistance (SAR) to pathogens can be artificially activated by exogenous application of JA and SA respectively and it is for this reason that researchers have long hoped that these compounds could be used as an environmentally benign method of crop pest control. However, in the last few years increasing evidence has emerged that indicate that the SA and JA pathways do not act independently of one another, rather they interact in a complex manner (Karban & Kuc 1999, Maleck & Dietrich 1999, Bostock *et al*. 2001), possibly allowing the 'fine tuning' of defence responses to multiple threats (Reymond & Farmer 1998). These interactions may be synergistic leading to the enhancement of responses (e.g. Schweizer *et al*. 1998), but are more often antagonistic where the efficacy of the response is reduced when several pathways are simultaneously involved, or if the other pathway has been previously activated (Felton *et al*. 1999, Bostock *et al*. 2001, Thaler 1999, Thaler *et al*. 2002b). In terms of applying this technology in commercial crop production these antagonistic interactions between the JA inducing pest and SA inducing disease resistance pathways produce an unacceptable level of uncertainty for growers. For this reason researchers have sought signal molecules that act to potentiate both these pathways without the associated problems of negative pathway interaction.

One potential candidate molecule that has recently been identified is the non-protein amino acid β-aminobutyric acid (BABA). The mechanism by which BABA induces

broad spectrum disease resistance is still somewhat unclear but there is mounting evidence that it is both necessary for plants to mount effective defences to a range of pathogens and when applied exogenously to foliar tissue can trick plants into activating the full range of disease defence mechanisms independent of both the JA and SA pathways. Recent work by in our laboratory has shown that JA can stimulate commercially relevant, long-term pest resistance when applied as a short, cost-effective seed treatment. Therefore the purpose of this study is to both investigate whether BABA, when applied as a seed treatment, induces resistance to powdery mildew (*O. neolycopersici*) in mature tomato and secondly to determine whether JA when applied simultaneously with BABA to seed interferes with that resistance.

Materials & Methods

BABA seed treatment

β-aminobutyric acid (BABA) was dissolved in distilled water only to a concentration of 3mM. Seed of tomato (*Lycopersicon esculentum* Mill cv. Carousel) were fully submerged in solution and stored at 4°C for a period of 24h before being thoroughly washed in distilled water only for approximately 5 mins before being germinated as described below.

JA seed treatment

Jasmonic acid (Sigma, Poole, UK) was dissolved in 100% ethanol before dilution with distilled water to a concentration of 3mM. Seed of tomato (*Lycopersicon esculentum* Mill cv. Carousel) were fully submerged in solution and stored at 4°C for

a period of 24h before being thoroughly washed in distilled water only for approximately 5 mins before being germinated as described below.

BABA and JA seed treatment

Jasmonic acid (Sigma, Poole, UK) was dissolved in 100% ethanol before dilution with distilled water to a concentration of 3mM. BABA was then added to this solution at a concentration of 3mM. Seed of tomato (*Lycopersicon esculentum* Mill cv. Carousel) were fully submerged in solution and stored at 4°C for a period of 24h before being thoroughly washed in distilled water only for approximately 5 mins before being germinated as described below.

Control seed treatment

Distilled water was substituted for JA in an ethanol control solution and was applied as above.

Plant material

Seeds from all treatment groups were sown in in 120 mm diameter pots in a controlled environment (CE) growth room, with a day / night temperature of 20 $\pm$ 2 °C / 16 $\pm$ 2 °C, a 14 h photoperiod and photosynthetically active radiation (PAR) of 300-350 μmol m^{-2} s^{-1}. Experiments began 85d after germination as detailed below.

Infection with *Oidium neolycopersici*

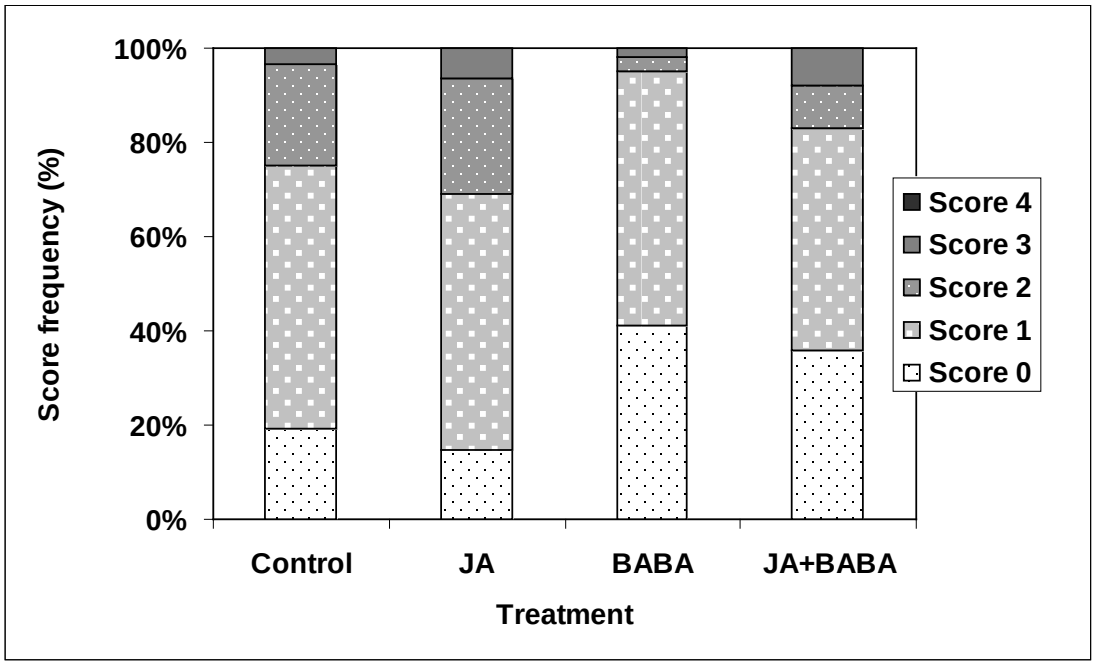
At 85d tomato plants were inoculated with by natural infection. Plants were placed in a heavily infested glasshouse compartment (where the pathogen is maintained on

tomato cv. Carousel) for natural infection by the pathogen. Individual mature leaves (three oldest leaves of tomato and five oldest leaves of tobacco) on each plant were evaluated for disease development at 20 and 35 days postinoculation by scoring severity on a scale of 0–4, where 0 = no symptoms, 1 ≤ 5% leaf surface covered with mycelium, 2 = 5–25% covered, 3 = 25–50% covered and 4 ≥ 50% covered. Five or six plants were used per treatment.

Results

By itself JA had no effect on the progression of disease symptoms when compared to untreated controls ($P>0.05$; Figs. 1.a & 1.b). In contrast, treatment with BABA only significantly reduced disease progression at both 20d ($P<0.05$; Fig. 1.a) and at 35d ($P<0.001$; Fig. 1.b). To test whether the observed protective effect of BABA was negatively affected by JA treatment (which we know from previous seed treatment experiments primes tomato to pest attack but is also a known antagonist of the disease resistance pathway), we challenged mature plants from simultaneously treated JA & BABA seed. JA & BABA treatment plants showed a similar reduction in disease severity to BABA only treatment group at both 20 ($P>0.05$; Fig. 1.a.) and 35 ($P>0.05$; Fig. 1.a.) days.

a)



b)

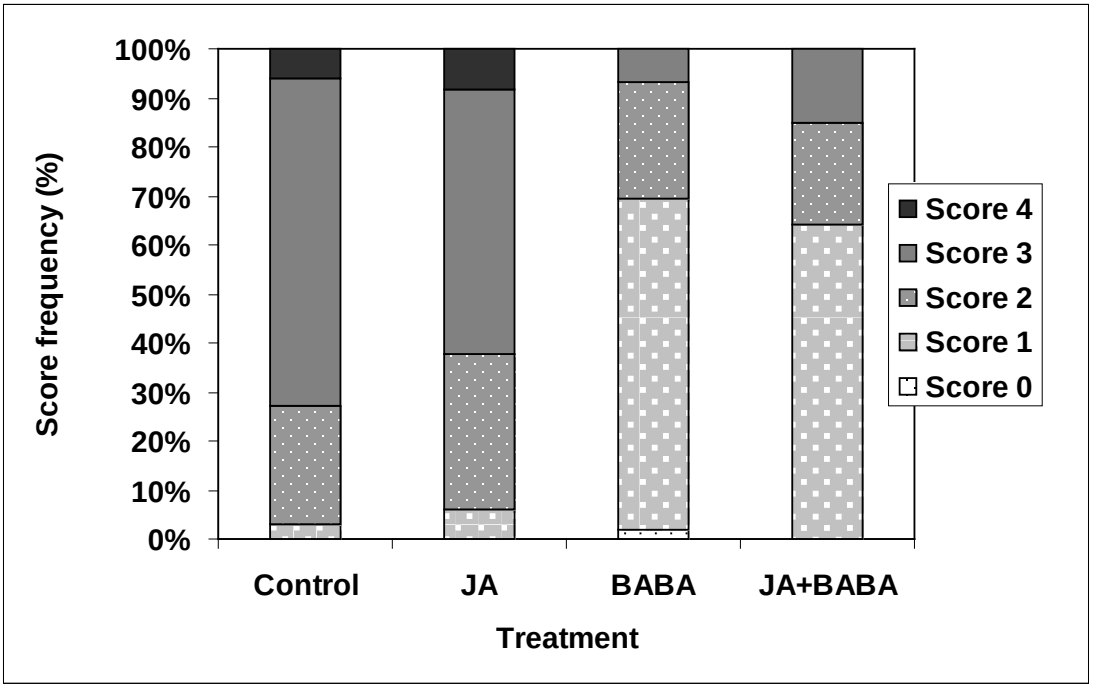


Figure 1. Effect of JA, BABA and JA+BABA seed treatment on disease severity of tomato against *O. neolycopersici*. Disease severity was scored at 20 and 35 days postinoculation using a standard 0–4 scale.

Discussion

A concern highlighted by previous studies which have attempted to simultaneously elicit both crop-plant pest (IR) and disease (SAR) resistance is that negative cross-talk between the corresponding pathways negate the beneficial affects observed when the pathways are attenuated independently. For this reason, and of course because of issues surrounding both economics and public safety linked to the spraying of such chemical elicitors on food crops, induced resistance technology has largely failed to make an impact on the way in which growers control economically important pests and disease.

The results from this study highlight a new aspect of the biological action of the non-protein amino acid BABA in priming tomato for resistance to a major global economic disease; powdery mildew. Furthermore, we show that commercially relevant, long-term reductions in disease progression can be achieved following a relatively short (24 hour) treatment of tomato at the seed stage and simultaneous treatment with JA for pest resistance does not significantly interfere with BABA attenuated resistance *for L. esculentum*. At 20 days post-inoculation approximately 40% of BABA and BABA+JA treatment plants showed no symptoms of disease compared to only 20% of JA and Control plants (Fig. 1.a). By 35d both JA and Control plants showed similar levels of disease progression with 60 and 70% of plants respectively scoring 3 (25–50% disease coverage) or 4 (≥ 50% coverage) (Fig.1.b). In contrast, disease progression was significantly reduced in both BABA and BABA+JA plants ($P<0.001$; Fig. 1.b). In both these treatment groups over 80% of plants scored between 0 and 2; which represents between $0 \leq 25\%$ disease coverage only (Fig. 1.b).

In all previous investigations we are aware of where JA and BABA have been used as inducers of resistance these compounds are generally applied as a foliar spray (Thaler et al 1999), root drenches (Bostock 1997) or are applied to detached leaf discs. Here we provide evidence that BABA enhances resistance through potentiation of pathogen-specific plant-defence responses, leading to a restriction of pathogen growth and spread when applied as a seed treatment. One possible mechanism by which BABA could act as an inducer of resistance might be through diffusion across microscopic ruptures caused by imbibition and the subsequent binding of BABA to specific receptors on the surface of individual and undifferentiated embryonic cells. As previously mentioned we have evidence that treatment of seed with JA imparts long-term (we have evidence for up to eight months at this stage) resistance to a range of pests across the globes major crop species (data not published for commercial reasons). The resistance potency of BABA has similarly been reported in a number of plant species including gravevines (Cohen, Reuveni & Baider 1999), pepper (Hong, Hwang & Kim 1999) and sunflower (Tosi, Luigetti & Zazzerini 1999) which opens up the possibility of its efficacy in a much wider range of crops.

Importantly, results from this study indicate that there is little or no negative cross-talk between the JA and BABA pathways; or at least JA does not inhibit BABA's ability to prime for disease resistance in tomato. This is an interesting observation since, when using foliar sprays at least, the activation of JA dependent defence responses has been shown to limit SAR driven pathogen resistance in tomato and other crop species (Felton *et al*. 1999, Bostock *et al*. 2001, Thaler 1999, Thaler *et al*. 2002b). Furthermore, in studies using grape leaves BABA induced resistance is linked to downstream activation of SA as a mediator of SAR induced resistance (Ton &

Mauch-mani 2004). One possible area of further study should focus on unraveling the BABA specific pathway and its relationship to the well characterized JA and SA mediated pathways.

This study adds to our understanding of the importance of induced defence responses in crop plants. Results presented here further highlight the possibility that BABA, when applied independently and possibly in conjunction with JA for induction of pest resistance, may provide a novel, cost-effective and environmentally benign method for the protection of the world's crop species from both major economic pests and disease.

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PART 5: Preliminary investigations into pigment production in lollo rosso (*Lactuca sativa*) under crop covers with different UV transmission properties.

Introduction

The pre-packed baby salad industry is worth approximately £650 million per year in the UK and growth is forecast to continue expanding as the trend for both healthy and convenient food continues. Consumption of a mix baby leaves with a combination of different colours and flavours is becoming the norm while purchase of single whole-head lettuce is increasingly the exception. Leaves are grown and harvested from locations across the world to provide the large-retailers with fresh, bagged, salad 365 days of the year.

Over the last four years lollo rosso, a major component of bagged salads for its colour and distinctive taste, has been grown under state-of-the-art crop covers at Stockbridge Technology Centre. The purpose of these studies has been to determine the effect on lollo rosso biomass accumulation and crop taste and quality of three of these covers; each possessing varying UVA and UVB transmission properties. As well as directly affecting the production of vegetative biomass by changing the characteristics of the expanding cell-wall exposure to UV light is also known to affect synthesis of colour pigments in the leaf tissue; a major quality component of lollo rosso.

In lollo rosso we have observed clear and consistent changes in the colouration of vegetative tissue in response to the different UV regimes under the three crop covers and this was thought to be directly linked to changes in the levels of flavonoids linked to colouration. In addition to acting as pigments in various crop tissues, flavonoids are known to be involved in a vast array of other biological functions in plants. Certain flavonoids play a role in plant stress defence responses, such as in protection against damage caused attacking pathogens and pests and in response to excess UV-light.

Flavonoids are strongly UV-absorbing compounds; with accumulation primarily occurring in epidermal cells of plant tissues after exposure to UV. Leaf epidermal layers have been shown to absorb as much as 90-99% of the incident ultraviolet radiation (Robberecht & Caldwell 1983) and their localisation in epidermal layers, coupled with their known ultraviolet absorptive properties, has led to a suggestion that they can serve primarily as protection against potentially harmful UV. Indeed there is a growing body of evidence for the role of flavonoids in photoprotection (Winkel-Shirley 2002) and several groups have reported changes in flavonoid composition of plant leaves as a consequence of excess light or UV-radiation (Lois 1994, Olsson *et al*. 1998, Hofmann *et al*. 2000, Tattini *et al*. 2000, Kolb *et al*. 2001).

The purpose of this preliminary study was to quantify the level of flavonoids in both double and triple red varieties of lollo rosso under three crop covers with diverse UV transmission properties.

Materials & Methods

Plant material.

Plants of commercial double and triple red lollo rosso lettuce (*Lactuca sativa* L. cv. Challenge, Syngenta seeds Ltd, UK) were raised for 14d from sowing using a widely employed UK commercial practice at Crystal Heart Salads (Holme-on-spalding-Moor, UK). Briefly, seeds were germinated in 4cm^3 peat blocks (Fison B2 Blocking Compost, Fisons, UK) at 16 $\pm$ 3°C in the dark for 4d before being transferred to commercial glass for a further 10d. At 14d plants were transferred to STC and randomly distributed under the three filter treatments for approximately eight weeks at which point they were harvested and processed for pigment analysis at Lancaster University..

*Crop-scale experiments: The facility at Stockbridge Technology Centre***.**

All crop-scale experiments were carried out at Stockbridge Technology Centre (STC: 53N 1W) using a series of commercial high-tunnel structures (Haygrove Tunnels Ltd., Ledbury, UK). Each spectral filter structure covers 740m^2 over four individual bays, each measuring 3 m high X 6 m long.

Plastics.

In our experiments we make use of a range of three commercially produced plastic cladding films (all supplied by Bpi.agri Ltd., Stockton-on-Tees, UK). In all cases the base film is 150-µm-thick polyethylene, with specific additives, conferring specific spectral transmission properties. The control film (Standard) is a standard commercial horticultural cladding film that had a PAR transmission of 93% when new. Transmission in the UV declines rapidly with decreasing wavelength from 90% at 400 nm to less than 10% below 350 nm. Total UV-A transmission is approximately 50%.

Transmission in the UV-B is less than 5% and effectively zero below 300 nm. Two films with modified UV transmission are used. The UV-opaque film has a total PAR transmission of 95% but a total UV-A transmission of only 10% and its UV-B transmission is zero. Transmission in the UV is zero below 375 nm but increased to around 60% at 400nm. The UV-transparent film has a transmission greater than 80% across the whole of the solar UV range from 290 to 400 nm. Total transmission in the PAR and the UV-A are 94% and 90% respectively.

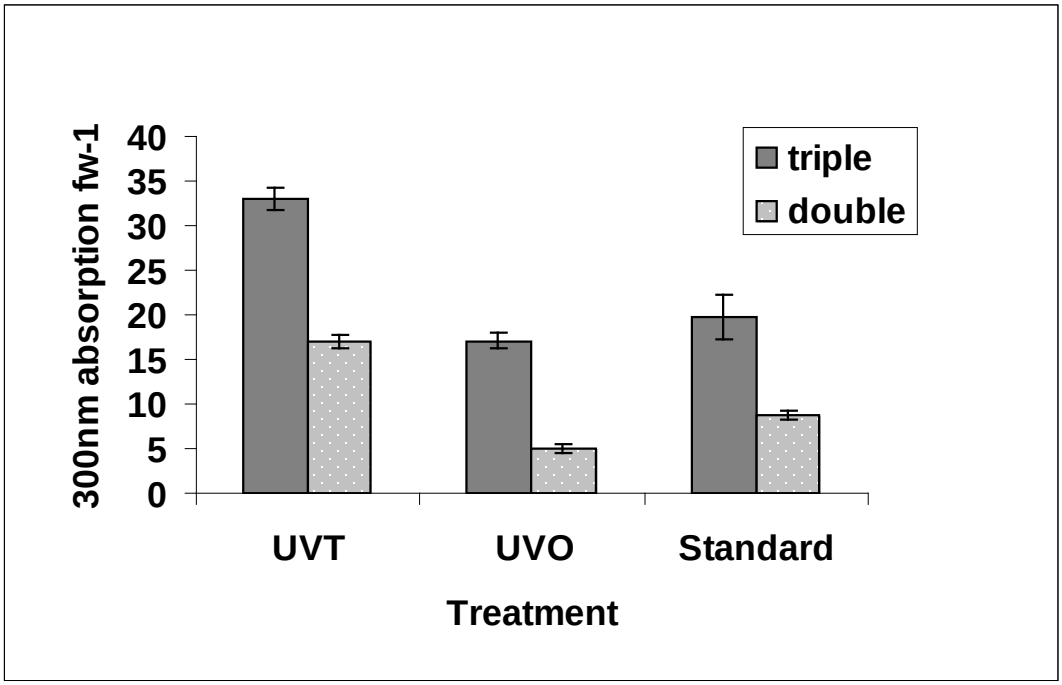
Results

To study the impact of solar UV-A and UV-B on the levels of total flavonoids we quantified these compounds at both 300 and 524 nm in both double and triple red lollo rosso varieties under either a UV-blocking, a UV-transparent and a Standard UK horticultural clear plastic films. At 300nm total flavonoids were significantly increased in triple-red crops grown under the UVT cover when compared to UVO ($P<0.001$; fig.1.a) and the Standard ($P<0.001$; fig. 1.a). Similarly, in the double-red variety total flavonoids were significantly increased in UVT relative to the remaining two treatments ($P<0.001$; Fig.1.a). Somewhat surprisingly there was a non-significant increase in total flavonoids in both double and triple red lollo rosso in Standard when compared to UVO ($P>0.05$; Fig. 1.a). Within each crop cover treatment group there was between a 46% (UVT) and 76% (UVO) increase in flavonoids measured in triple red variety compared to the double red variety (Fig. 1.a.).

At 524 nm UVT produced exhibited increased flavonoid production in both double and triple red lollo rosso compared to UVO ($P<0.001$; Fig. 1.a) and Standard ($P<0.001$; Fig 1.b). Similar to results at 300 nm Standard produced marginal (non-

significant) increases in double and triple red flavonoids relative to their respective UVO crops (Fig. 1.b). Within each crop cover treatment group flavonoids increased by 74% (UVT), 83% (UVO) and by 66% in Standard (Fig.1.b).

a)



b)

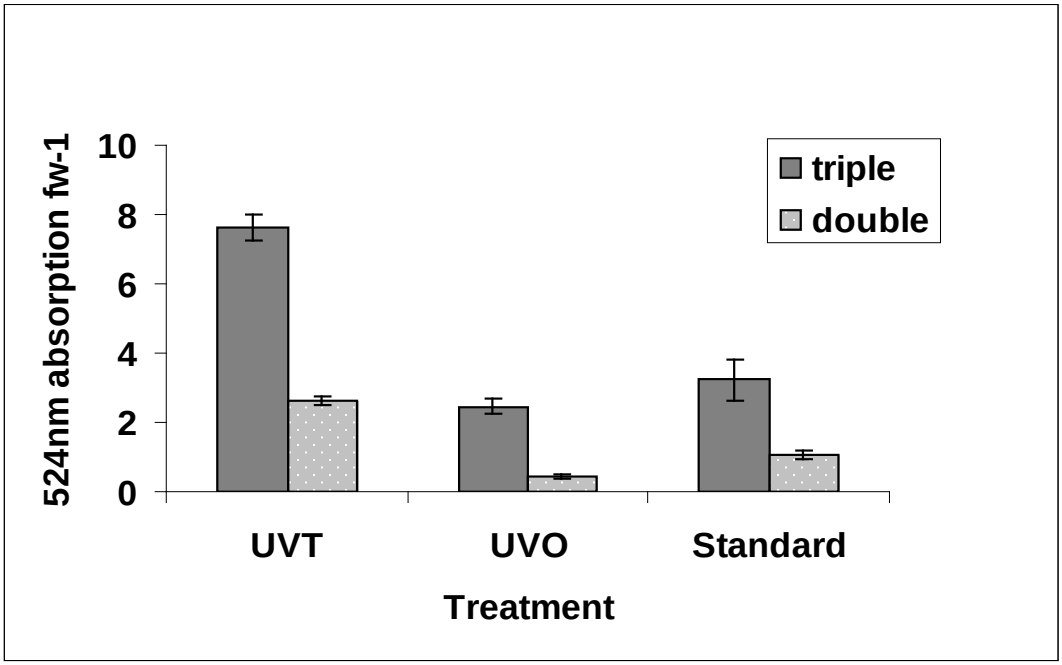


Figure 1. Total flavonoids measured in both double and triple red lollo rosso varieties at a) 300 nm and 524 nm

Discussion

Lollo rosso is primarily used in mixed leaf pillow packs and so the visual properties of the crop are of great importance; with high level of red pigmentation in leaf tissue being a desirable trait. Over at least three plantings per year across 4 UK growing seasons we have visually observed that lollo rosso produced under a largely UV-transparent crop cover has consistently produced lollo rosso crops with commercially relevant increases in pigmentation.

Results from this preliminary study; the first quantification of both double and triple red varieties of lollo rosso bulk flavonoids, from crops produced under three UV manipulating crop covers show significant relative increases in their synthesis under the filter most transparent to solar UV (Figs. 1.a. & b). In both the double and triple red varieties exposure to high ambient solar UV increased bulk flavonoids measured at both 300 and 524 nm and these increases were in the order of at least 50%.

Flavonoids comprise the most common group of polyphenolic plant secondary metabolites. In crop plants, flavonoids play an central role in various biological processes; they function as pigments in flowers and fruits, attract pollinators and seed dispersers, directly affect resistance to disease and are thought to serve as UV-scavengers. The first direct evidence in support of a role for flavonoids in UV protection came from experiments with *Arabidopsis* mutants, which showed that lesions in chalcone synthase (CHS) or chalcone isomerase (CHI) resulted in UV-hypersentive phenotypes (Li et al 1993). The flavonoids reduce the damage from UV-B radiation because they act as UV filters, reducing the penetration of potentially

damaging UV-B radiation (Santos et al. 2004). Importantly, results from this study provide further evidence of a causal relationship between increasing crop exposure to UV and increased flavonoid synthesis possibly as a mechanism for UV protection (Fig. 1.a. & b).

Because these compounds underpin such a diverse range of plant functions and responses to environmental stress they are found in a wide variety of fruits and vegetables and therefore form an integral part of the human diet. In addition to the well-established antioxidant activity of many of these compounds *in vitro*, an inverse correlation between the intake of certain polyphenols and the risk of cardiovascular disease, cancer and other age related diseases has been observed in epidemiological studies (Hollman et al 1999). For these reasons there is growing commercial interest in the effects of dietary polyphenols on human health. At a time when large retailers are focusing on adding value to their product range and consumers are demanding healthier alternatives in convenience foods one exciting possibility could be the production of fresh bagged salads with high UV induced flavonoid content.

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