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Project number:	Project FV 386
Project leader:	Shreya Wani, Newcastle University
Report:	Annual report (Year 1), September 2012
Previous report:	N/A
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AUTHENTICATION

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

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CONTENTS

GROWER SUMMARY

Headline

In vitro studies suggest microorganisms associated with post-harvest spoilage are sensitive to ozone gas, but in vivo studies have not demonstrated a significant reduction of microbial load.

Background

The shelf-life of the fresh leafy produce is affected by the presence of spoilage microbes. Also, where field crops are sprayed with pesticides, residue issues maybe an issue for growers. Hence, there is a need to develop residue free alternatives to reduce both microbial load and pesticide residue levels; particularly in the climate of potential changes in EU pesticide regulation. Successful development of a residue free crop storage method will enhance the healthy image of the sector, enhance marketability of fresh produce and improve sales. The technology should create a market lead for UK produce and develop an expertise base in the UK.

The aims of the year 1 work were to explore the potential afforded by ozone treatment to reduce post-harvest spoilage and extend the shelf-life of leafy salads and herbs and specifically:

- To characterize microbial population over the shelf-life of targeted leafy produce
- To determine the impacts of ozone on key elements of the surface microflora
- Optimise the concentration & duration of ozone exposure for fresh and processed product
- Visualize bacteria on spinach leaves by using Confocal Scanning Laser microscopy.

Summary

This project focuses on the use of gaseous ozone treatment administered during pre-packaging to reduce post-harvest contamination, spoilage and pesticide residue levels in targeted produce (leafy salads and root vegetables). Initial work has focused on laboratory and pilot-scale optimization of ozone exposure treatments (level*duration) to reduce spoilage and enhance shelf-life of leafy salads. Depending on findings, the study may be extended to explore the impacts of ozone treatment on pesticide residues and/or a wider range of produce (especially root vegetables). It is anticipated that commercial-scale trials will be conducted during the course of the study to test the efficacy of the technology in an industry environment.

Objective 1: To characterize the principle microbial population of shelf-life of targeted leafy produce

Two packets of organic Italian style salad (lettuce, wild rocket & spinach), watercress & rocket, organic spinach & coriander were purchased and the microflora over the shelf-life of the product examined. Products were stored at 4^{o}C in the dark as directed on the packaging and tested at the start of life (SOL) and sell-by-date i.e. end of life (EOL). A range of micro-organisms were present and microbial numbers ranged from 10^5 to 10^9 CFU/g. An increase in microbiological counts was observed as the duration of incubation increased (i.e. with the passage of shelf-life). The most numerous microbial genera were *Pseudomonas spp., Debaryomyces spp. and Cryptococcus spp.*

Objective 2: To determine the ozone sensitivity of key classes of microbial shelf-life determinants in artificial culture

Pseudomonas spp., Yeasts and Moulds were isolated from Coriander samples and cultured on agar plates. These plates were then exposed to 1, 10, & 50 ppm ozone or 'clean air' (controls) for 10 minutes. The number of colonies propagated on control plates (non-ozone exposed) was compared to the numbers found on ozone-treated plates. Colony numbers (CFU) of *Pseudomonas fluorescens* (bacterial model species), *Alternaria alernata* (fungal model species) and *Debaryomyces hansenii* (yeast model species) *in vitro* were significantly reduced by ozone treatment. Direct ozone-treatment of plates prior to introduction of microbes delivered similar results. There was no significant difference between the treatments so direct impacts of ozone on the media used to culture the organisms was not the reason for the observed effects (data shown in the full report).

Objective 3: To optimise the concentration and duration of ozone exposure for fresh and processed leafy product

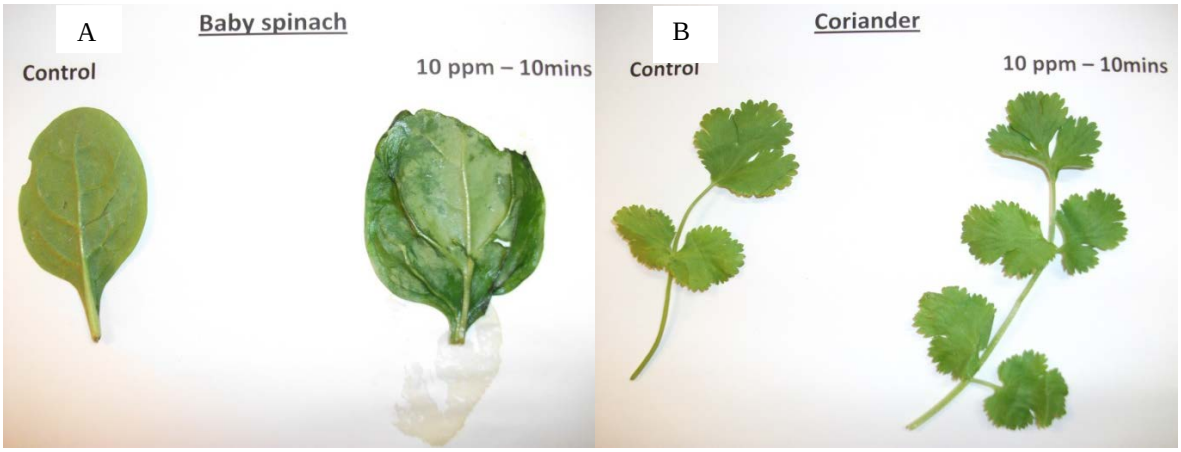
No visual ozone damage was observed when leafy produce was exposed to 1 ppm ozone for shorter time periods.

Impact of high ozone concentration on visual quality of fresh produce

Target produce	**Concentration (ppm)**	**1**						**10**		
	Time (ppm.min)	**0.18**	**2.30**	**7.26**	**12.26**	**27.29**	**57.89**	**1.93**	**21.02**	**69.88**
Watercress		**+**	**+**	**+**	**−**	**−**	**−**	**−**	**−**	**−**
Baby spinach		**+**	**+**	**+**	**+**	**+**	**−**	**−**	**−**	**−**
Coriander		**+**	**+**	**+**	**+**	**+**	**−**	**+**	**+**	**+**
Lettuce		**+**	**+**	**+**	**+**	**+**	**−**	**−**	**−**	**−**

Key: '**+**' indicates positive effect/no visible damage on target produce when exposed to particular ozone concentration. '**−**' indicates negative effect/visible damage on target produce when exposed to particular ozone concentration

In contrast, only coriander retained its appearance when exposed to 10 ppm ozone for any significant period of time (see the photograph below). Ozone injury/visible damage was observed on all produce when exposed to 25 and 50 ppm ozone concentration suggesting there is no 'window of opportunity' at these concentrations.



Impacts of gaseous ozone (10 ppm) on A) baby spinach and B) coriander when exposed for 10 minutes

Objective 4: To explore the ozone sensitivity of microbes present on surface of leafy produce in vivo

Packs of coriander were exposed to either 10 ppm ozone or 'clean' air for 10 minutes and then maintained at 4°C in cold storage room (dark conditions). The total viable count of *Pseudomonas spp*, Yeasts and Moulds were made on day 0 (labeled as 'SOL' – start of life) and on the sell-by-date (labeled as 'EOL' – end of life). The total viable count, were enumerated after growth on agar plates.

There was no significant reduction in Total Viable Counts (TVC), *P. fluorescens,* moulds and yeasts after exposure of fresh produce to similar ozone treatments as the in vitro studies described in Objective 3, where significant reductions in microbial load could be seen. This is an interesting observation and suggests strongly that there are fundamental differences between the phenotype of the microbes under investigation in culture and on the surface of produce.

The next stage of this work will seek to clarify the hypothesis that *P. fluorescens* is in fact the key determinant of the shelf-life of leafy salads and herbs. Future work will also explore, in some depth, the nature of the difference in sensitivity to ozone of *P fluorescens in vivo* and *in vitro*. It will attempt to probe the physiological and biochemical basis for the differential sensitivity to ozone.

Financial Benefits

At present it is too early in the project to deliver financial benefits. Some very interesting results on the apparent resistance of spoilage bacteria to ozone exposure on a leaf surface mean that fundamental work is required to dissect the observation. This understanding may lead to methods that can overcome resistance. An alternative approach may be the manipulation of the microbial leaf population to reduce spoilage. Such methods could deliver clear financial benefits for the leafy salads industry.

SCIENCE SECTION

Introduction

Field crops suffer from post-harvest microbial contamination and decay. Previous work has demonstrated that long-term exposure to low atmospheric concentrations of ozone can be effective in some crops (e.g. berries and citrus) in significantly reducing mould proliferation (Barth *et al*., 1995; Sarig *et al*., 1996; Palou *et al*., 2003; Tzortzakis *et al*., 2007 a,b).

Ozone is well known for its strong oxidizing capacity and has been recognized for over a century as a powerful antimicrobial agent, reacting with organic substances approximately 3,000 times quicker than chlorine (Singh *et al*., 2002). The gas has been widely used to treat both drinking water and effluent (Perez *et al*., 1999). Research into the antimicrobial effects of ozone have identified significant differences in the response of spores, mycelia and Gram positive and Gram negative organisms (Guzel-Seydim *et al*., 2004; Singh *et al*., 2002). In 1997, the United States Food and Drug Administration (US-FDA, 2008) granted ozone GRAS (Generally Recognised as Safe) status (Graham *et al*., 1997) and later, in 2003, it received formal approval from the US-FDA as a 'direct contact food sanitizing agent' (Karaca and Velioglu, 2007). Current European regulations enable the administration of ozone where national health and safety regulations relating to worker exposure are strictly adhered to. This has led to commercial interest in the exploitation of ozone for numerous industrial applications including, but not restricted to, waste water treatment, surface disinfection, sanitization of equipment, ozone injection into storage areas and modified atmosphere packaging (Tzortzakis *et al*., 2007a, b). One of the major advantages of ozone treatment is the fact the gas leaves no detectable residues in/on treated products as ozone rapidly decomposes into oxygen (Guzel-Seydim *et al*., 2004).

Aim of work conducted so far:

To explore the potential afforded by ozone treatment to reduce post-harvest spoilage and extend the shelf-life of leafy salads and herbs

- To characterize microbial population over the shelf-life of targeted leafy produce
- To determine the impacts of ozone on key elements of the surface microflora
- Optimise the concentration & duration of ozone exposure for fresh and processed product
- Visualize bacteria on spinach leaves by using Confocal Scanning Laser microscopy.

Materials and methods

The principal microbial population present on leafy salads

Two packets of organic Italian style salad (lettuce, wild rocket & spinach), watercress & rocket, organic spinach & coriander were purchased and the microflora over the shelf-life of the product examined. Products were stored at 4^{o}C in the dark as directed on the packaging and tested at the start of life (SOL) and sell-by-date i.e. end of life (EOL). Using ISO/British Standard methods produce was stomached and plated to enable enumeration of TVCs (Total Viable Counts), Yeasts and Moulds (CFU/g). Discrete colonies of the dominant microbial types were subsequently recultured and characterized using 16/28S DNA sequence analysis.

Preliminary test to determine the ozone sensitivity of key classes of microbes isolated from fresh produce

Pseudomonas spp., Yeasts and Moulds were isolated from Coriander samples and cultured on Cephaloridin Fucidin Centrimide (CFC) and Dichloran Rose Bengal Chloramphenicol (DRBC) agar plates. These plates were then exposed to 1, 10, & 50 ppm ozone or 'clean air' (controls) for 10 minutes. The number of colonies propagated on control plates (non-ozone exposed) was compared to the numbers found on ozone-treated plates.

This experiment focused on optimizing the concentration and duration of ozone exposure to which fresh produce could be exposed without causing visible damage/deterioration. To determine the impact on visual quality of the produce, fresh leafy produce were received from Intercrop Ltd. and Vitacress ltd. and then exposed to 1, 10, 25, 50 ppm ozone or 'clean air' (controls) for varying periods of time (ppm.min; ozone concentration*duration). Following exposure to ozone produce was then packed in a sealed plastic bag (maintained at 4°C in dark conditions). Ozone injury was assessed visually by comparing with control produce.

Exploration of the 'ozone hardiness' of unwashed fresh produce

To explore whether unwashed fresh produce was more ozone resistant than washed produce, 'unwashed' fresh produce was received direct from Spanish subsidiaries of Intercrop Ltd. and Vitacress Ltd. and treated as previously described.

Impacts of high ozone concentration on microbial flora present on surface of leafy salads

Eighteen packages of coriander were purchased and the total viable count of each was assessed at day 0 (labeled as 'SOL' – start of life) and on the sell-by-date (labeled as 'EOL' – end of life) by exposing these produce to either 10 ppm ozone concentration or 'clean' air for 10mins and then maintained at 4°C in cold storage room (dark conditions). At SOL and EOL, total viable count,

Pseudomonas spp, Yeasts and Moulds were enumerated after growth on Cephaloridin Fucidin Centrimide (CFC) or Dichloran Rose Bengal Chloramphenicol (DRBC) agar plates.

To determine impacts of pre-exposure of media to ozone on subsequent colony development in vitro

To examine whether *in vitro* versus *in vivo* differences in microbial ozone sensitivity represent an anomaly due to the experimental protocol adopted in *in vitro* investigations, additional CFC and DRBC plates were pre-exposed to either 10 ppm ozone or 'clean' air for 10 minutes before carrying-out the inoculation step as mentioned in the earlier section.

Visualization of bacteria on spinach leaves as demonstrated by Confocal Scanning Laser microscopy using BacLight LIVE/DEAD Assay stains

A packet of organic baby spinach was purchased from a local retailer and maintained in cold room at 4°C till the use-by-date labelled as 'EOL' (end of life). The leaves were then aseptically cut into pieces measuring approximately 1 cm by 1 cm and placed onto sterile glass slides. The staining procedure was performed using the LIVE/DEAD® BacLight™ Viability Kit (Invitrogen/molecular probes) as per the manufacturer's instructions. This protocol utilises green-fluorescent SYTO®9 stain to label all bacterial cells green, whereas all dead cells were stained red by red-fluorescent propidium iodide stain. Hence, live cells stained green and dead cells stained red. The staining solutions were prepared in Mueller Hinton Broth (MHB) and filter-sterilized using a syringe-mounted membrane filter of 0.2 µm pore size prior to use. The stains were applied directly to the leaf surface in 250µl aliquots using Gilson style pipette, a coverslip was placed on top of the stained leaf. The stained leaf was then incubated in the dark for 30 minutes prior to viewing using Confocal Scanning Laser Microscopy (CSLM). Images were captured at 60x magnification, using an immersion oil lens.

Statistical analysis

Data were analysed using SPSS (IBM SPSS Statistics 19 64Bit) and graphs were produced using Microsoft Office Excel 2010. Significant differences between mean values were verified using LSD ($P < 0.05$) following one-way ANOVA.

Results

Microbial populations over the shelf life of leafy salads

The range of micro-organisms present, and the microbial counts (which ranged from 10^5 to 10^9 CFU/g), is shown in Table 1. An increase in microbiological counts was observed as the duration of incubation increased (i.e. within the shelf-life time frame). The most numerous microbial genera were *Pseudomonas spp., Debaryomyces spp. and Cryptococcus spp.*

Table 1: The genera of microbiota present over the shelf life of packed leafy produce with their total viable count (CFU/g) at 'SOL' – start of life and 'EOL' – end of life

Specimen sample	Counts (CFU/g)		Bacteria	Counts (CFU/g)		Fungal
	SOL	EOL		SOL	EOL	
Coriander	2×10^6	2×10^9	*Pseudomonas* *Bacillus*	1.4×10^5	9×10^6	*Cryptococcus* *Debaryomyces* *Cladosporium*
Organic spinach	1.6×10^8	2×10^8	*Pseudomonas* *Flavobacterium*	1.8×10^6	8×10^7	*Penicillium* *Cryptococcus* *Phaesophaeria* *Debaryomyces*
Rocket/ watercress	1.6×10^7	5×10^7	*Pseudomonas* *Aeromonas*	1.8×10^5	2×10^6	*Cryptococcus* *Debaryomyces*
Lettuce, wild rocket & spinach	1×10^7	3×10^8	*Pseudomonas* *Aeromonas* *Exiguobacterium*	1.4×10^5	9×10^6	*Cryptococcus* *Cladosporium* *Debaryomyces*

Preliminary test to determine the ozone sensitivity of the key classes of microbes isolated from fresh produce

Colony numbers (CFU) of produce derived *Pseudomonas fluorescens* (bacterial model species), *Alternaria alernata* (fungal model species) and *Debaryomyces hansenii* (yeast model species) *in vitro* were significantly reduced ($P < 0.05$) by ozone treatment (Figure 1), even at extremely low levels (1 ppm)

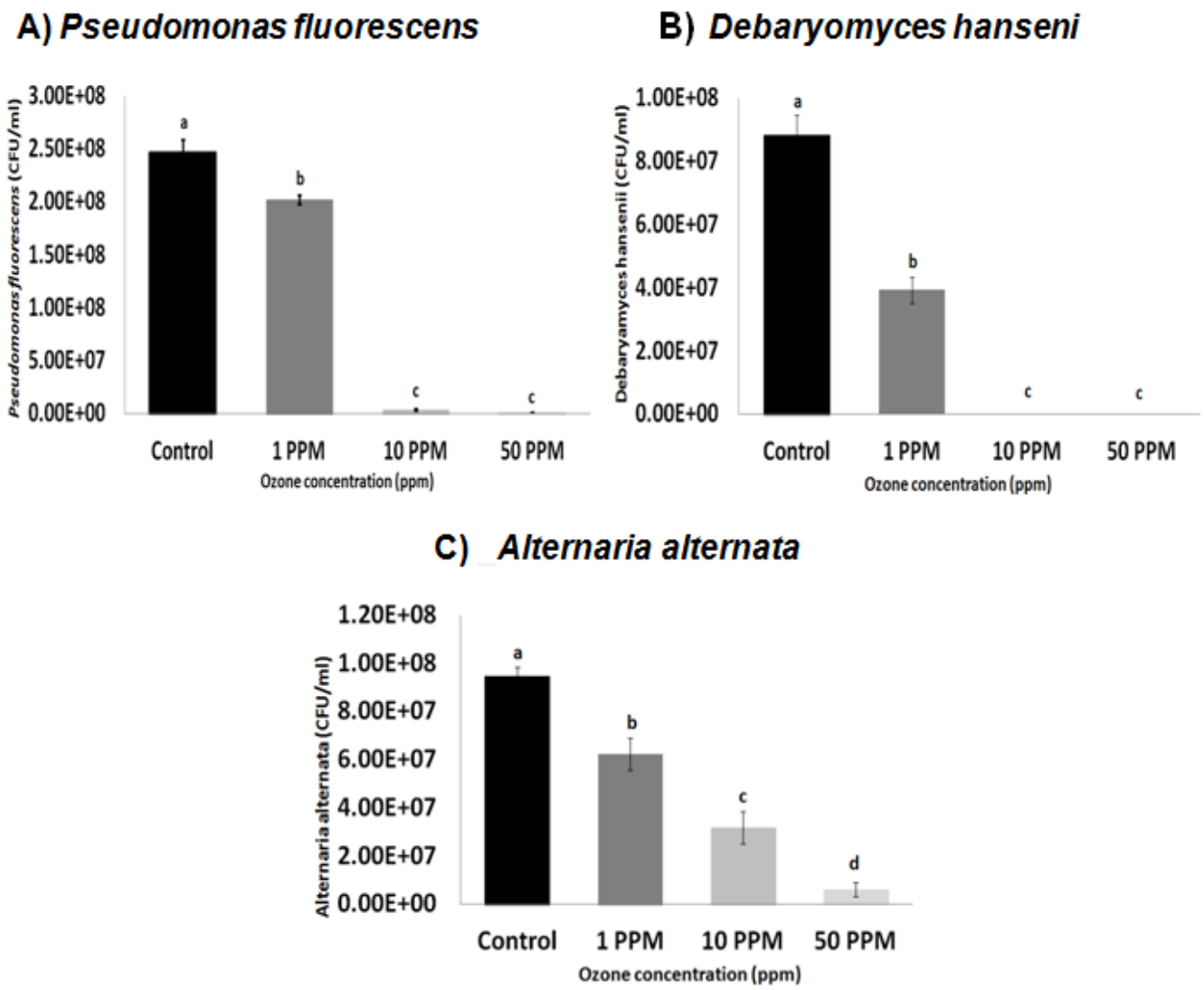


Figure 1: Impacts of ozone-enrichment on A) *P. fluorescens* exposed to 10ppm ozone on CFC plates while B) *D. hansenii* and C) *A. alternata* exposed to 10ppm ozone on DRBC plates (CFU/ml). The treatment chamber was ventilated with 1, 10 or 50 ppm ozone. Controls were subjected to 'clean air'. Values represent the mean (±Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

Ten minutes' exposure to ozone concentrations of 10 ppm and 50 ppm resulted in a highly significant reduction in counts of all the tested microbes; *P. fluorescens, A. alternata* and *D. hansenii*.

Exploration of the 'ozone hardiness' of washed EOL product

No visual ozone damage was observed when leafy produce was exposed to 1 ppm ozone (Table 2) for the shorter time periods. In contrast, only coriander retained its appearance when exposed to 10 ppm ozone for any significant period of time. Ozone injury/visible damage was observed on all produce when exposed to 25 and 50 ppm ozone concentration suggesting there is no 'window of opportunity' at these concentrations (data not shown).

Table 2: Impact of high ozone concentration on visual quality of fresh produce

Target produce	Concentration (ppm)	1						10		
	Time (ppm.min)	0.18	2.30	7.26	12.26	27.29	57.89	1.93	21.02	69.88
Watercress		+	+	+	−	−	−	−	−	−
Baby spinach		+	+	+	+	+	−	−	−	−
Coriander		+	+	+	+	+	−	+	+	+
Lettuce		+	+	+	+	+	−	−	−	−

Key: '**+**' indicates positive effect/no visible damage on target produce when exposed to particular ozone concentration. '**–**' indicates negative effect/visible damage on target produce

Exploration of the 'ozone hardiness' of unwashed fresh produce

The unwashed' fresh produce was sourced straight from the field. Results suggested unwashed produce may be slightly hardier, as no visible injury was observed on produce exposed to 10 ppm for 1.9 ppm.min (approx. 5 minutes) (Table 3).

Table 3: Impact of high ozone concentration on visual quality of 'unwashed' fresh produce

Target produce	Concentration (ppm)	1						10		
	Time (ppm.min)	0.18	2.30	7.26	12.26	27.29	57.89	1.93	21.02	69.88
Watercress		+	+	+	+	−	−	+	−	−
Baby spinach		+	+	+	+	+	−	+	−	−
Coriander		+	+	+	+	+	−	+	+	+

Key: '**+**' indicates positive effect/no visible damage on target produce when exposed to particular ozone concentration '–' indicates negative effect/visible damage on target produce

Impacts of ozone treatment on microbial flora (present on surface) of leafy salads

Exposure of produce to similar ozone treatments resulting in positive effects when microbes were treated on plates (Figure 1) interestingly resulted in no significant reduction ($P < 0.05$) in counts of TVC, *P. fluorescens,* moulds and yeasts (Figure 2).

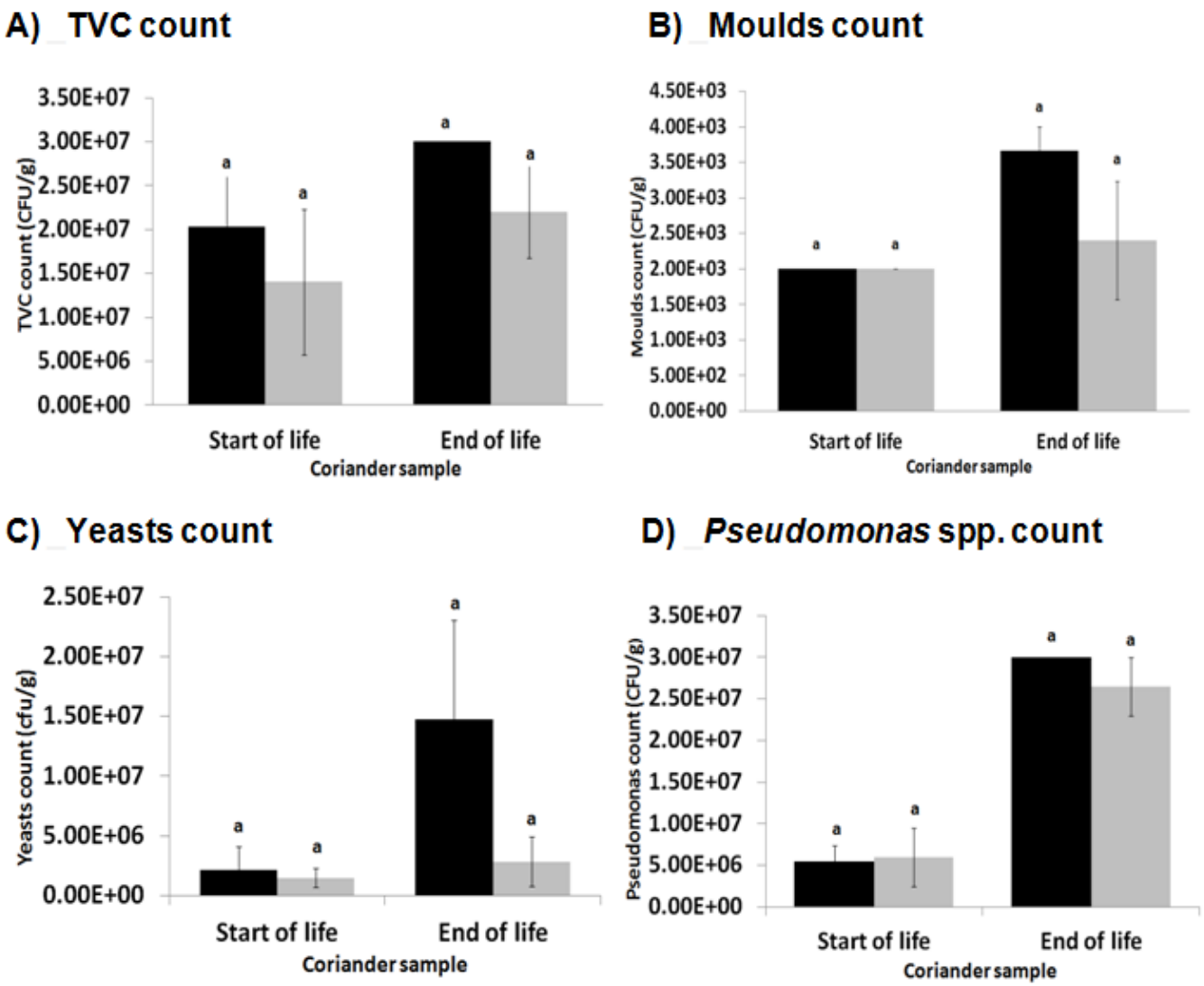


Figure 2: Impacts of ozone-enrichment on microbial flora present on surface of coriander. Produce were either exposed to 10 ppm ozone concentration (grey bar) or 'clean' air (black bar) for 10 minutes. Values represent means (±Standard Error) of measurements made on three independent packets of coriander per treatment. Bars with different letters are statistically significantly different (ANOVA, $P < 0.05$).

The impacts of pre-exposure of media to ozone on subsequent colony development in vitro

Direct ozone-treatment of plates prior to introduction of microbes delivered similar results. There was no significant difference between the treatments ($P < 0.05$) so direct impacts of ozone on the media used to culture the organisms was not the reason for the observed effects (Figure 3).

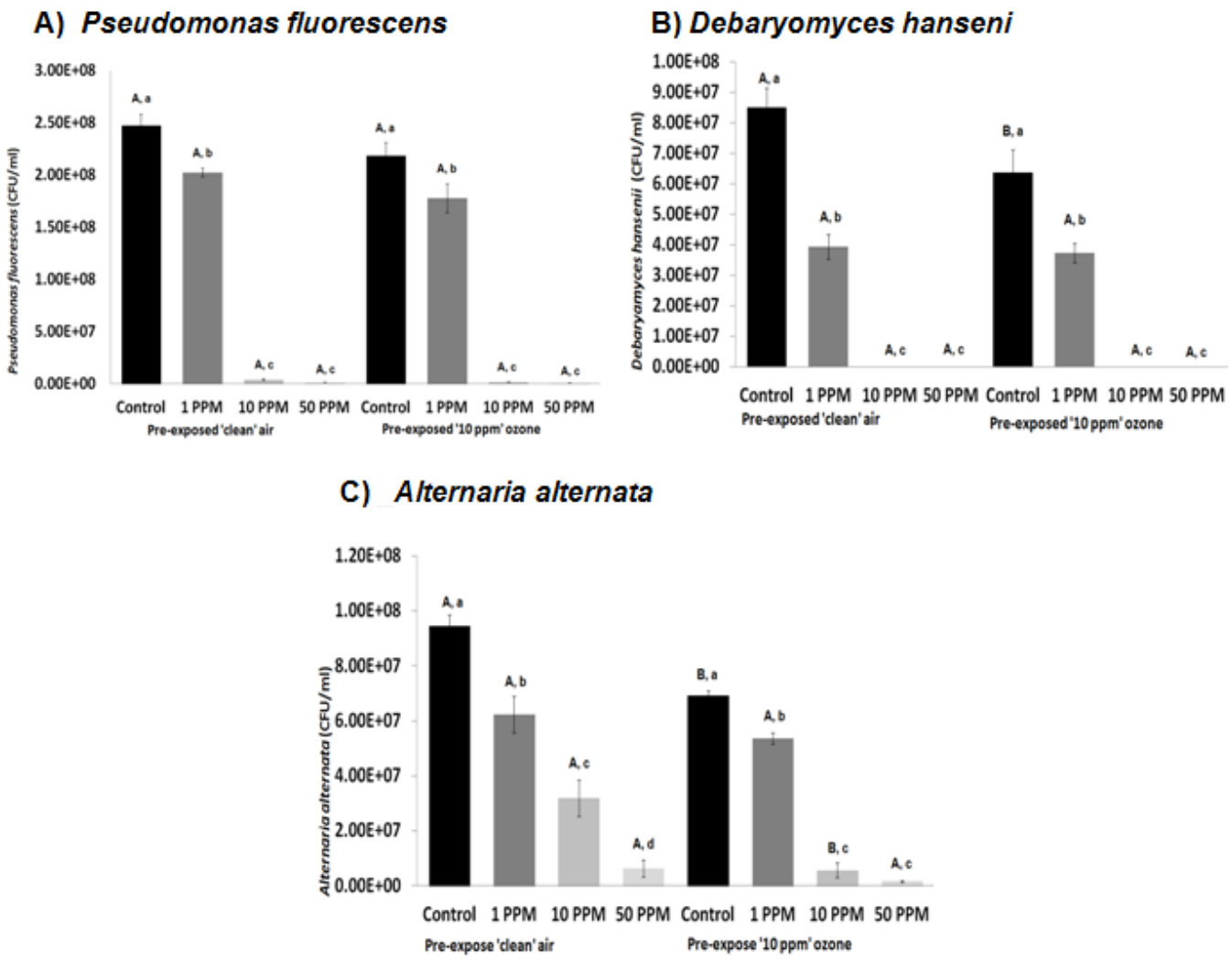


Figure 3: Impacts of ozone-enrichment on A) *P. fluorescens* exposed to ozone on CFC plates while B) *D. hansenii* and C) *A. alternata* (CFU/ml) exposed to ozone on DRBC plates. Plates were either pre-exposed to 10 ppm ozone or 'clean air' for 10 minutes. The treatment chamber was ventilated with 1, 10 or 50 ppm ozone. Controls were subject to 'clean air'. Values represent the mean (±Standard Error) of measurements made on three independent plates per treatment. Bars on the graph within ozone level and pre-exposed treatment with the same letter are not statistically significantly different (ANOVA, $P < 0.05$).

Visualization of bacteria on spinach leaves

Bacteria were observed attached mainly to the leaf epidermal cell margins (Figure 4) and stomata of spinach leaf. These bacteria also seemed to gather inside the stomata rather than penetrating the leaf through stomata.

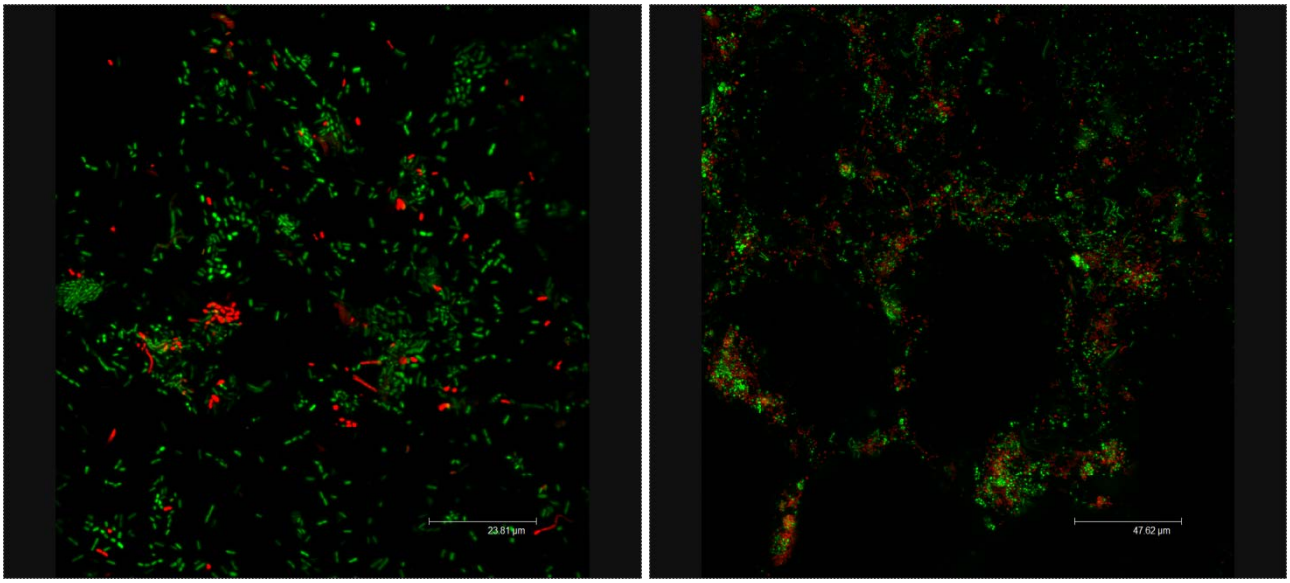


Figure 4: Confocal microscopy image of the baby spinach leaf. (A) Bacteria were stained with green-fluorescent SYTO®9 to label live bacterial cells green and with red-fluorescent propidium iodide to label dead bacterial cells red. Scale bar = 23.8 µm (B) the bacteria appeared to attach preferentially to the epidermal cell margins. Scale bar = 47.6 µm

Discussion

Not unexpectedly (Lindow *et al*., 2003) leafy salads and herbs were found to be colonized by a variety of bacteria, yeasts and moulds. The microbes ranged in number from 10^5 to 10^9 CFU/g. The most numerous microbial genera were *Pseudomonas fluorescens, Alternaria alernata* and *Debaryomyces hansenii,* with the principal determinant of shelf-life of the packed product believed to be *Pseudomonas fluorescens.* This is consistent with Morris and colleagues' (1997) conclusions that *Pseudomonas fluorescens* is the principle determinant of the shelf-life of leafy salads and herbs.

Three microbes isolated from produce: *Pseudomonas fluorescens, Alternaria alernata* and *Debaryomyces hansenii* were exposed to ozone in an *in vitro* experiment. All were inactivated by 10 minutes exposure to ozone concentrations above 10 ppm.

No visual ozone damage was observed when leafy produce were treated with 1 ppm gaseous ozone, but only coriander retained its freshness when exposed to 10 ppm ozone. Damage on the leafy produce was observed when high ozone dosage was applied.

Ozone exposure did not reveal any added benefits on 'unwashed' fresh produce. It was thought that stomatal conductance could be lower (and more controllable) in unwashed produce – but this did not seem to be the case *via* provisional investigation. Further work will be conducted measuring stomatal opening using a porometer and attempts will be made to manipulate stomatal conductance to enhance the hardiness of the crop to ozone treatment using modified atmosphere regimes.

In contrast to the ozone-induced reduction in microbial counts observed *in vitro*, equivalent ozone exposure resulted in no effects when produce was treated *per se*. This is an interesting observation and suggests strongly that there are fundamental differences between the phenotype of the microbes under investigation in culture and on the surface of produce. The next stage of this work will seek to clarify the hypothesis that *P. fluorescens* is in fact the key determinant of the shelf-life of leafy salads and herbs. Future work will also explore, in some depth, the nature of the difference in sensitivity to ozone of *P fluorescens in vivo* and *in vitro*. We will attempt to probe the physiological and biochemical basis for the differential sensitivity to ozone.

In vivo bacterial viability was demonstrated by using LIVE/DEAD BacLight Viability kit which contains mixtures of SYTO 9, a green fluorescent nucleic acid stain, and PI, the red fluorescent nucleic acid stain. This method uses the green fluorescent stain to penetrate the intact cell membrane as well as cells with damaged membranes and red fluorescent stain which only penetrate damaged membrane (Molecular Probes, Inc., 1996). This stain combination produced a clear colour difference which allows us to distinguish between live and dead cells.

Confocal Scanning Laser Microscopy (CSLM) has shown that the common sites for bacterial aggregation of leaf surfaces are the cell wall margin and stomata. This observation is supported by the data of Romantschuk *et al*., 1996 who observed attachment of bacteria to leaf surfaces is mostly at the cell wall junctions.

The bacteria are capable of secreting carbohydrate polymers known as exopolysaccharides (EPS) which form capsular layer when combined with the cell wall (Leigh & Coplin, 1992). *Pseudomonas* spp. known to colonize leaf surface are capable to produce EPS. The EPS layer provides protection to microbes from environmental stresses by retaining moisture and absorbing nutrients from plant surface. These microbial aggregates with EPS are referred to as biofilms (Carmichael *et al*., 1999). The current study of biofilm formation on the leafy produce is still at an early phase of development. Further study is needed to investigate the potential that microbes on the surface of leafy produce are protected from ozone by a biofilm formation and factors influencing biofilm with regards to product quality by using confocal microscopy and appropriate staining techniques.

Conclusions

- *Pseudomonas spp., Debaryomyces spp.* and *Cryptococcus spp.* appear to be the principle determinants of shelf-life of the herbs and leafy salad products screened to date
- Short-term-exposure to high ozone concentrations is capable of inactivating principle elements of the surface microflora
- Treatment regimes have been identified that may – in theory – be commercially applicable to achieve effective microbial decontamination without damage to the produce *per se.* Work in Year 2 will focus on understanding how the 'sensitivity' of microbes varies *in vitro* and *in vivo* as it appears that those on the surface of the product maybe considerably more resistant to ozone treatment than would be predicted from *in vitro* investigations

Knowledge and Technology Transfer

Site visit to Vitacress to understand production and processing of leafy salads, and explore opportunities for technology transfer.

Networking at HDC Student Conference 2012.

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Appendices

Raw data for Preliminary test to determine the ozone sensitivity of microbes isolated from fresh produce

Pseudomonas	Pre-exposed non-ozonated air for 10 mins		
	Replicate I	Replicate II	Replicate III
Control	251000000	226000000	265000000
1 PPM	197000000	200000000	211000000
10 PPM	3000000	3000000	6000000
50 PPM	0	1000000	2000000

Yeasts	Pre-exposed non-ozonated air for 10 mins		
	Replicate I	Replicate II	Replicate III
Control	99000000	89000000	77000000
1 PPM	33000000	47000000	38000000
10 PPM	0	0	0
50 PPM	0	0	0

Moulds	Pre-exposed non-ozonated air for 10 mins		
	Replicate I	Replicate II	Replicate III
Control	93000000	89000000	102000000
1 PPM	60000000	75000000	52000000
10 PPM	33000000	20000000	43000000
50 PPM	5000000	12000000	2000000

Raw data of the impacts of ozone treatment on microbial flora (present on surface) of leafy salads

Aerobic TVC at 30°C	control 1	control 2	control 3	ozone 1	ozone 2	ozone 3
coriander UB 24.02.12 SOL	10000000	30000000	21000000	2100000	10000000	30000000
coriander UB 24.02.12 EOL+3 days	30000000	30000000	30000000	30000000	24000000	12000000

Moulds	control 1	control 2	control 3	Ozone 1	ozone 2	ozone 3
coriander UB 24.02.12 SOL	2000	2000	2000	2000	2000	2000
coriander UB 24.02.12 EOL+3 days	4000	3000	4000	1200	2000	4000

Yeasts	control 1	control 2	control 3	ozone 1	ozone 2	ozone 3
coriander UB 24.02.12 SOL	418000	6000000	100000	1090000	3000000	400000
coriander UB 24.02.12 EOL+3 days	30000000	12800000	1450000	455000	7000000	1070000

Pseudomonas	control 1	control 2	control 3	ozone 1	ozone 2	ozone 3
coriander UB 24.02.12 SOL	1820000	6600000	8000000	2000000	2980000	13000000
coriander UB 24.02.12 EOL+3 days	30000000	30000000	30000000	19500000	30000000	30000000

Raw data of the impacts of pre-exposure of media to ozone on subsequent colony development *in vitro*

Pseudomonas	Pre-exposed non-ozonated air for 10 mins			Pre-exposed to 10PPM Ozone for 10 min		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	251000000	226000000	265000000	195000000	220000000	240000000
1 PPM	197000000	200000000	211000000	179000000	201000000	153000000
10 PPM	3000000	3000000	6000000	0	3000000	1000000
50 PPM	0	1000000	2000000	0	1000000	1000000

Yeasts	Pre-exposed non-ozonated air for 10 mins			Pre-exposed to 10PPM Ozone for 10 min		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	89000000	89000000	77000000	77000000	51000000	63000000
1 PPM	33000000	47000000	38000000	39000000	31000000	42000000
10 PPM	0	0	0	0	0	0
50 PPM	0	0	0	0	0	0

Moulds	Pre-exposed non-ozonated air for 10 mins			Pre-exposed to 10PPM Ozone for 10 min		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	93000000	89000000	102000000	70000000	72000000	66000000
1 PPM	60000000	75000000	52000000	57000000	54000000	50000000
10 PPM	33000000	20000000	43000000	1000000	10000000	6000000
50 PPM	5000000	12000000	2000000	2000000	2000000	1000000