Project title:	Use of gaseous ozone to prevent microbial postharvest spoilage and reduce pesticide residue levels
Project number:	Project FV 386
Project leader:	Shreya Wani, Newcastle University
Report:	Annual report (Year 2), September 2013
Previous report:	Annual report, September 2012
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Date project commenced:	1st October 2011
Date project completed (or expected completion date):	30th September 2014

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

Exposure to ozone reduces *E. coli* and spoilage bacteria viability on salad leaves. Cold stress, such as that imposed by cold storage, may increase bacterial resistance to ozone.

Background

Pesticide resistance issues and consumer pressures over the use of pesticides have led to the exploration of residue free alternatives such as the use of ozone treatments to:

- reduce decay caused by spoilage microbes and;
- prevent microbial contamination of fresh produce after harvest.

The development of residue free pesticide alternatives will enhance the image of the sector, enhance the marketability of fresh produce and improve sales. This project aims to develop ozone treatments for the reduction of microbial spoilage and microbial contamination of leafy salad crops.

Summary

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 but less work has been done on leafy produce. This project focuses on the use of gaseous ozone treatment to reduce postharvest contamination and spoilage of leafy salads. Previous results from this work demonstrated that short-term exposure to high ozone concentrations was capable of reducing the viability of typical surface microflora found on produce *in vitro*. Current work focused on confocal scanning laser microscopy to visualize the effect of ozone exposure on natural microbial flora present on the leaf surface. In ozone-treated material significant inactivation of bacterial cells was observed by direct microscopic count of viable cells and the result was confirmed by traditional colony enumeration on plates. Interestingly we have found that both increasing cell age and stress (exposure to cold conditions) of a typical spoilage bacterium (*Pseudomonas fluorescens*) increases bacterial resistance to ozone exposure. In addition the impact of ozone exposure on the pathogen *Escherichia coli* inoculated onto spinach leaves was assessed and a significant reduction in cell counts was found following ozone treatment. Further work will focus on a) investigating the mechanisms of bacterial resistance to ozone using *P. fluorescens* as a model; b) developing ozone exposure protocols for a variety of leafy produce types that could potentially be trialed in a commercial setting.

Confocal microscopy: Visualization of bacteria on spinach leaves

Spinach leaves were observed using confocal scanning laser microscopy in conjunction with LIVE/DEAD® BacLight™ Viability Kit (Invitrogen/molecular probes) to see if the bacteria that survived ozone treatment were typically in colonies or individual cells. The large aggregation of live cells stained green is visible in Figure 1 (A) indicated by the blue arrow, at x63 magnification, scale bar = 23.8µm. Lots of individual small colonies and cells in twos or threes indicated by the yellow orange arrows (Figure 1A) respectively were observed. The bacteria mainly appeared to be rod shaped. Bacteria in chains indicated by the red arrow were also visible. Individual dead cells stained red are visible in Figure 1 (A) indicated by the white arrow. Figure 1 (B) shows bacteria were attached mainly to the leaf epidermal cell margins, observed at x63 magnification, scale bar = 47.6 µm. Similar bacterial aggregates were also observed on watercress, coriander, rocket and lettuce leaf surfaces (data not shown).

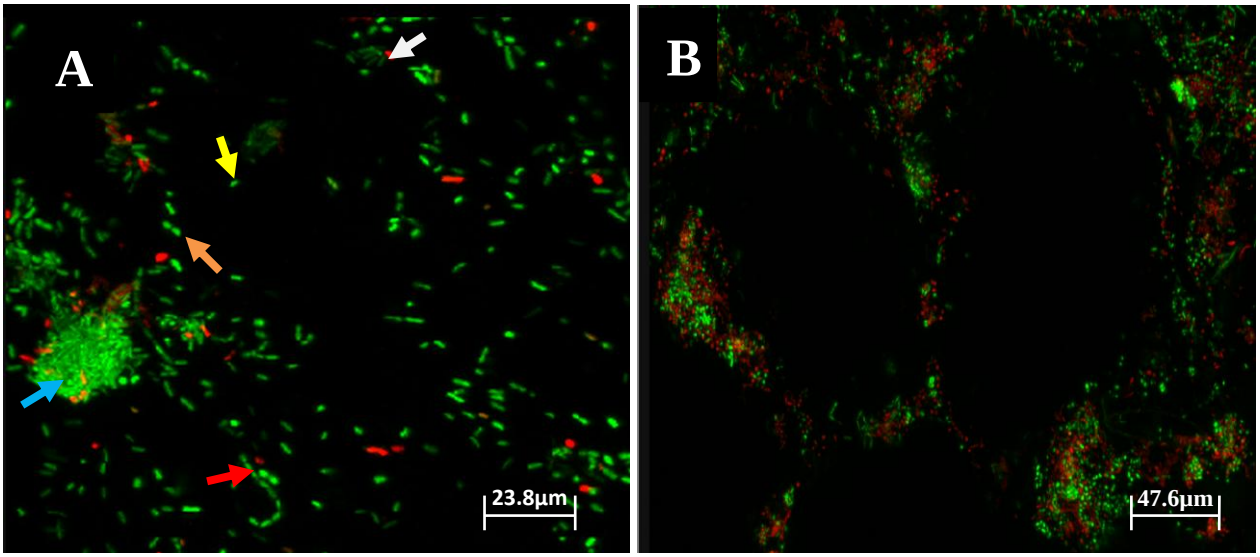

Figure 1: Confocal microscopy image of a baby spinach leaf. (A) Bacteria were stained with green-fluorescent SYTO®9 to label live bacterial cells green and with red-fluorescent propidium iodide to label dead bacterial cells red. Scale bar = 23.8 µm (B) the bacteria appeared to attach preferentially to the epidermal cell margins. Scale bar = 47.6 µm

Bacterial viability on leafy produce after ozone treatment: a comparison of direct microscopic count and indirect plate count methods

Using direct confocal microscopic observation on non-ozone exposed leaves (control), bacteria viability was nearly 90% (Figure 2A), whilst only 20% of bacteria on ozone treated leaf surfaces appeared viable. This reduction in bacterial viability was significant (P = 0.001). The yellow arrow (Figure 2B) indicates individual bacteria surviving ozone treatment while the white arrow (Figure 2B) indicates two/three live cells in a micro-colony of dead cells surviving ozone treatment. Plate counts also confirmed that microbial numbers on spinach leaf were significantly reduced (P = 0.000) by

ozone treatment (Figure 2A). Similar results were also observed on watercress, coriander, rocket and lettuce leaf surfaces (Figure 3).

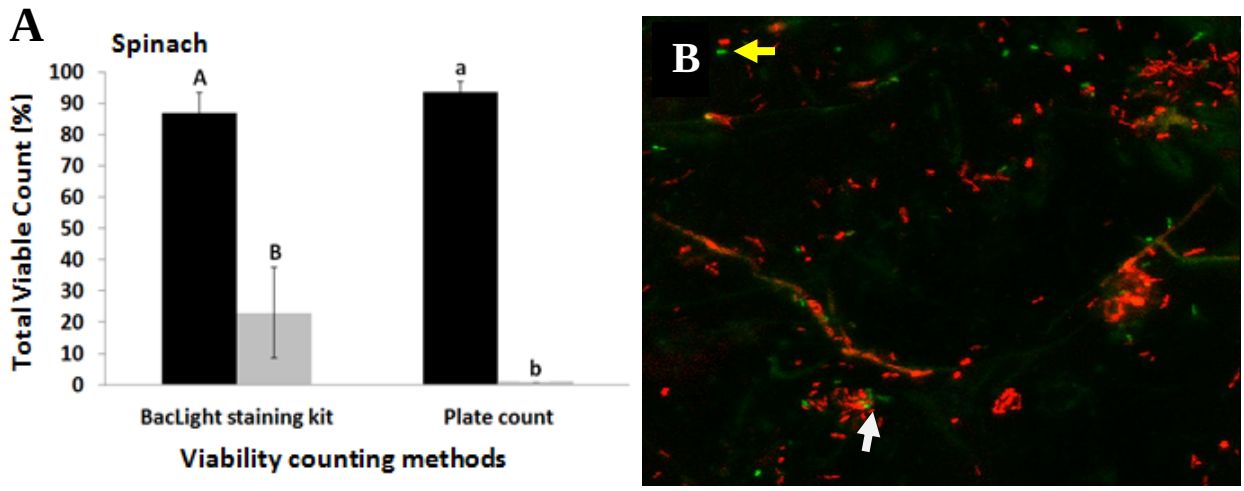

Figure 2: (A) Total viable count (%) of spinach leaves when treated either with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 10 minutes obtained using either direct microscopic counting of SYTO®9/PI stained bacteria on leaves and culture of bacteria on Plate count agar. Values represent means (+/- Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

(B) Confocal microscopy image of the baby spinach leaf when treated with 1 ppm ozone concentration for 10 minutes. 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.

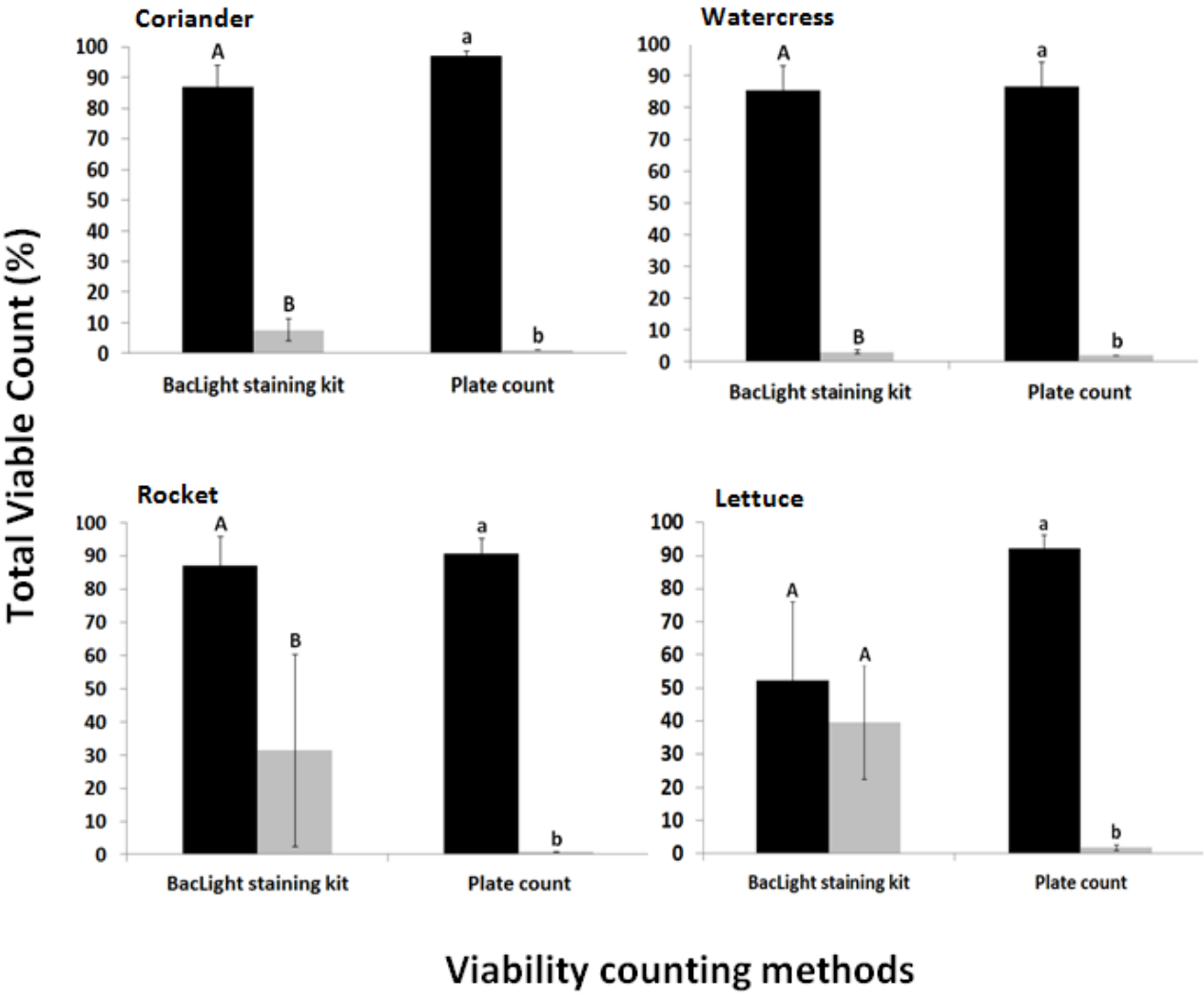

Figure 3: Total viable count (%) of coriander, watercress, rocket & lettuce leaves when treated either with 1 ppm/10ppm ozone concentration (grey bar) or untreated (black bar) for 10 minutes obtained using either direct microscopic counting of SYTO®9/PI stained bacteria on leaves and culture of bacteria on Plate count agar. Values represent means (Standard Error) of measurements made on three independent leaves per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

Effect of temperature on ozone resistance of P. fluorescens in vitro

Images from confocal microscopy of ozone treated leaves revealed that two or three live cells survived in micro-colonies surrounded by dead cells and also individual cells survived, indicating ozone resistance. To find potential reasons for this, the effect of temperature (mimicking cold storage conditions) on ozone resistance of *P. fluorescens in vitro* was studied as it is known that resistance to one stress factor can increase resistance to another stress factor.

Colony numbers (CFU) of *Pseudomonas fluorescens* maintained in optimum conditions (25°C) *in vitro* (i.e. where bacteria were grown and exposed to ozone on CFC plates) were significantly (ANOVA, $P < 0.05$) reduced by ozone treatment (Figure 4). In contrast, the colony numbers of *P. fluorescens* maintained in cold conditions (i.e. storage temperature 4°C) *in vitro* were not significantly (ANOVA, $P < 0.05$) reduced by ozone enrichment (Figure 4) indicating that cold stress enhances ozone resistance of the leaf surface bacteria.

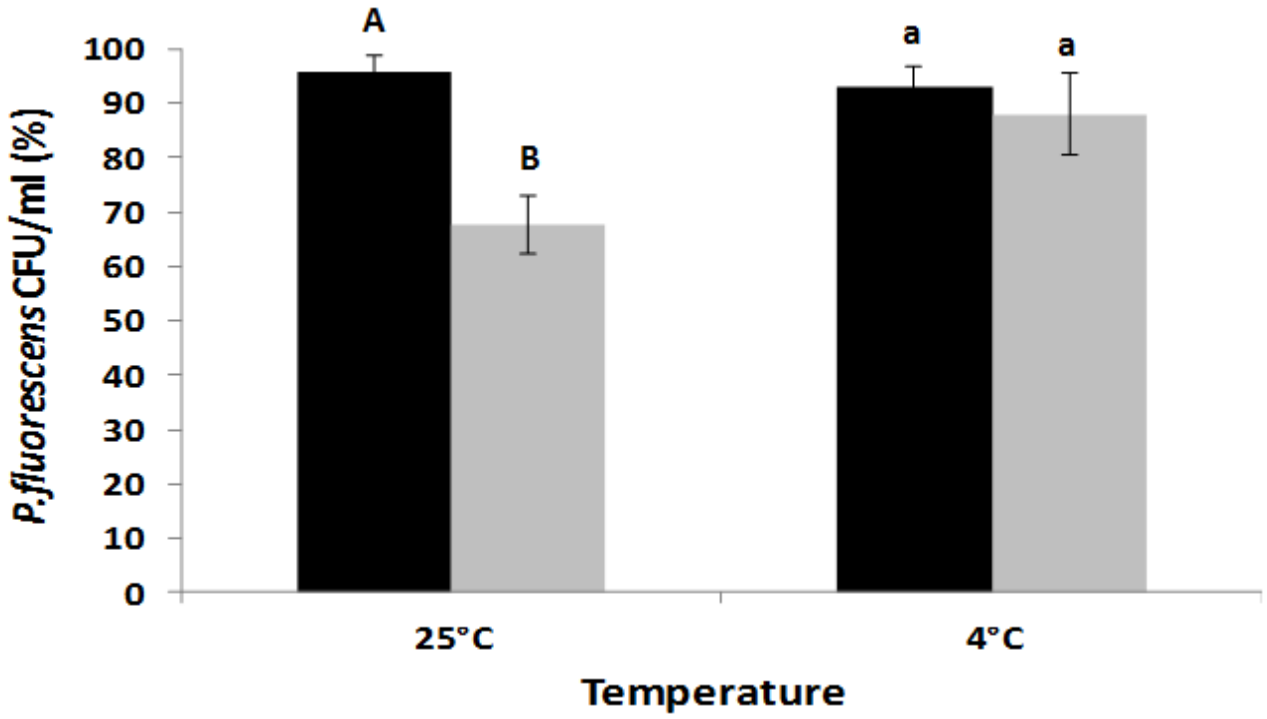

Figure 4: Impacts of ozone-enrichment on *P. fluorescens* grown at 25°C and 4°C and then exposed to either 1 ppm ozone concentration (grey bar) or 'clean' air (black bar) for 10 minutes. After treatment the plates were either incubated at optimum temperature i.e. 25°C or maintained in cold storage conditions at around 4°C. Values represent means (±Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

Effect of age of culture of P. fluorescens on ozone resistance

Separate cultures of *P. fluorescens* were grown for different lengths of time before exposure to ozone. After ozone treatment, survival of *P. fluorescens* (*in vitro*) was observed to be greater after 7 days of growth compared to day 2 and day 4 and this increased level of survival was maintained at day 10 and day 12 (Figure 5) suggesting that older bacteria are more ozone resistant than younger cells.

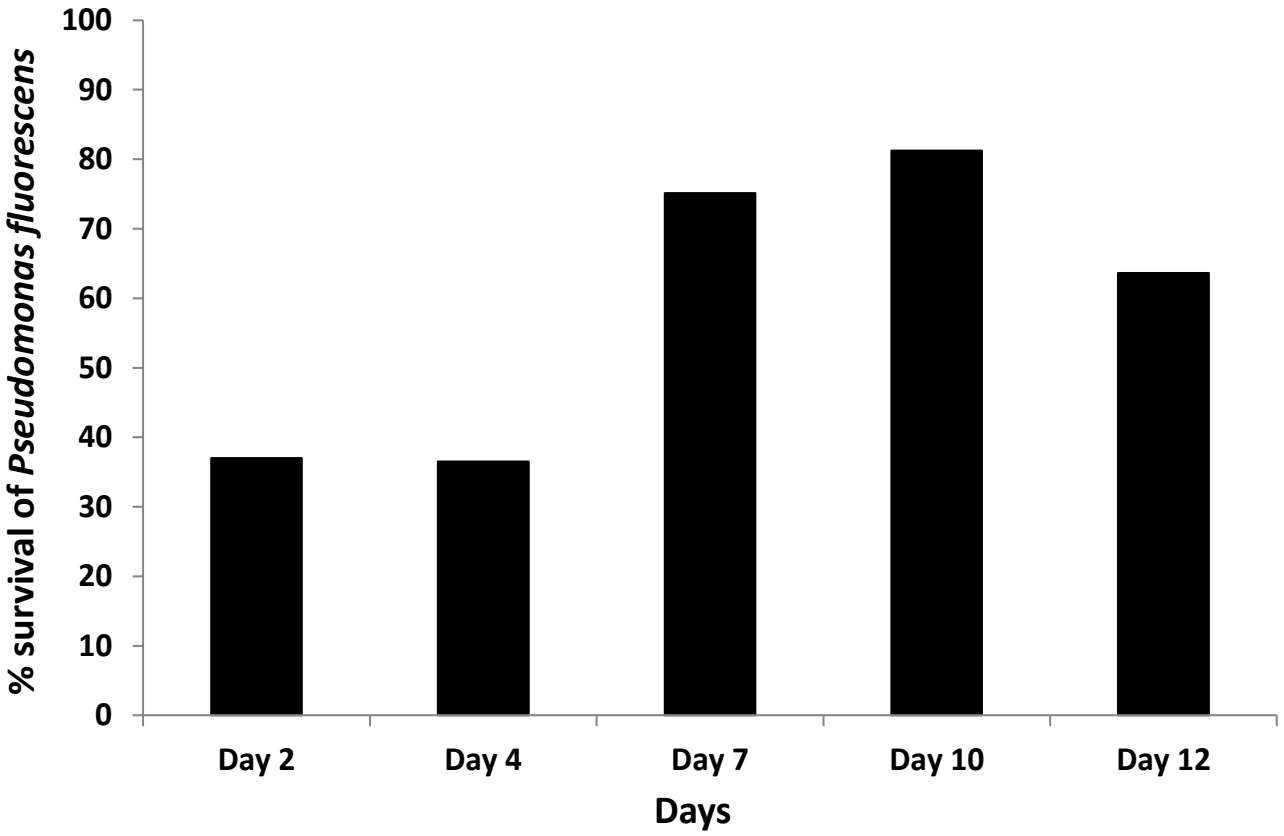

Figure 5: Impacts of ozone-enrichment on *P. fluorescens* exposed 1 ppm ozone concentration for 10 minutes. Culture plates were maintained in optimum temperature i.e. 25°C for 12 days. Values represent means (±Standard Error) of measurements made on three independent plates per treatment.

Effect of ozone exposure on E. coli strains inoculated onto spinach leaf surfaces

Colony numbers (CFU) of all six strains of *E.coli* i.e. *E.coli* O157:k88a, *E.coli* O25:h4, *E.coli* O128:k67, *E.coli* K12, *E.coli* O55:K59 and *E.coli* O104:h12 obtained from ozone exposed leaves were significantly reduced ($P < 0.05$) compared to non-ozone exposed controls (Figure 6). No *E.coli* colonies were isolated from non-inoculated spinach leaves. The bactericidal effect increased with ozone concentration, and length of ozone exposure (results not shown).

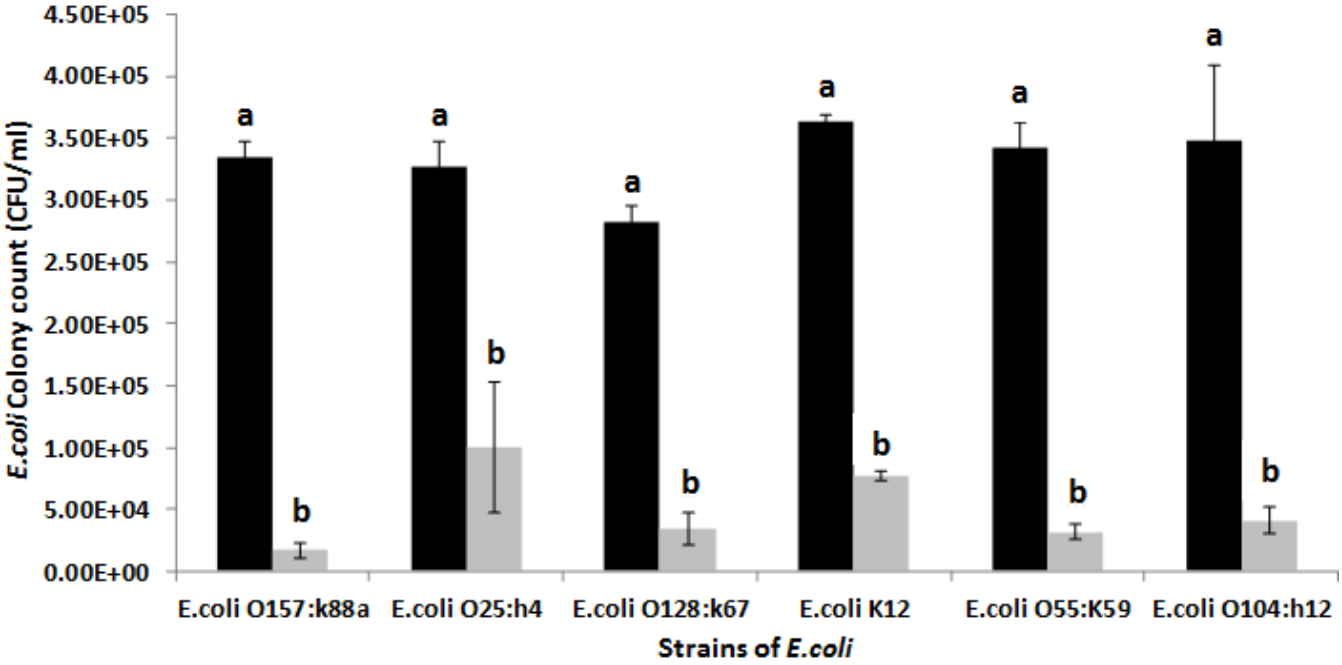

Figure 6: Impacts of ozone-enrichment on six strains of *E.coli* inoculated onto the surface of spinach leaves. Leaves were either treated with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 1 minute. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

Financial Benefits

None to date but experimental results so far indicate that ozone exposure decreases the number of spoilage bacteria and *E.coli*. Exposure treatments that do not destruct the leaf material have been identified. Further work is ongoing.

SCIENCE SECTION

Introduction

The microbial communities found on leafy produce are commonly dominated by bacteria from the *Enterobacteriaceae* and *Pseudomonadaceae* families (Nguyen-the *et al*., 1994 and Jacques *et al*., 1995) and the total microbial population ranges from 10^5 – 10^7 CFU/cm^2 (Carmichael et al., 1999). Generally the microbial populations present do not affect the leaf in the field but after harvesting and storage certain bacteria such as *Pseudomonas* spp., *Bacillus* spp. and *Erwinia* spp. can begin to cause soft rot of the produce (Carmichael *et al*., 1999). The microbial proteolytic and pectinolytic activities that cause soft rot can be carried out by these microbes at storage temperatures as low as 0.2°C (Magnuson *et al*., 1990). In addition to produce spoilage, microbes present on leafy produce have also been implicated in human disease. For example, leafy produce may be contaminated with food-borne pathogens, such as *Escherichia coli*, due to the application of animal manure fertilizer, contaminated irrigation water or deposition of faeces by livestock (Seo *et al*., 1998). *E.coli* can cause life threatening haemorrhagic colitis and hence is considered as a potential health hazard if present in or near raw produce (Cooley *et al*., 2007).

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). In 1997, the United States Food and Drug Administration (US-FDA) in union with an expert panel granted ozone as 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). 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). However, bacteria on fresh produce are not completely removed after ozone treatment (results from PhD year 1). This could be possibly due to a combination of physical protection of cells present in a micro-colony and increased expression of stress resistance genes in certain cells.

Microbial colonization of plant surfaces can be investigated using conventional transmission electron microscopy and scanning electron microscopy (Seo *et al*., 1998). However, the dehydration and fixation techniques involved in sample preparation result in artifacts which present a serious limitation to this conventional methodology (Little *et al*., 1991). This problem can be partially overcome by using confocal scanning laser microscopy (CSLM) as samples can be observed in a completely hydrated state (Seo *et al*., 1998). The adoption of CSLM in this study allowed the visualization of the bacterial colonies on the surface of the leaves *in vivo* and the exploration of whether the bacteria form a biofilm or exist as small colonies that may provide physical protection

from ozone treatment. It also provided an opportunity to compare direct counts of dead cells (by staining and direct visualisation) with indirect approaches (i.e. traditional viable count of cells recovered on plates).

Aim of PhD year 2:

a) To observe bacterial colonies and bacterial cell viability on leafy produce in response to ozone treatment using confocal scanning laser microscopy and compare results those obtained by traditional plate counts.

b) To determine if cell age and pre-exposure to stress (cold stress) affect ozone resistance of a typical spoilage bacterium (*P. fluorescens*)

c) To determine the ozone sensitivity of artificially inoculated *E.coli* on spinach leaves

Objectives

To develop a CLSM technique to visualize natural bacterial flora on leaves

To develop a method for differential staining of living and dead bacterial cells to evaluate the efficacy of ozone treatment on leafy produce

To study the effect of temperature and age on ozone resistance of the *P. fluorescens in vitro*

To study the impact of ozone treatment on artificially inoculated *E.coli* on spinach leaves

To assess if different *E. coli* strains exhibit differential sensitivity to ozone

Materials and methods

Leafy produce

Packets of organic baby spinach, Iceberg lettuce, wild rocket, coriander and watercress were purchased from a local retailer and stored at 4°C until the use-by-date i.e. 'EOL' (end of life). The leaves were then aseptically cut into pieces measuring approximately 1 cm by 1 cm and into discs using a sterile cork borer measuring 1.13 cm^2 for visualization of cell viability and enumeration of natural flora bacteria experiments, respectively.

Bacterial staining and visualisation for cell viability assessment – spinach leaves

Cell viability stains (LIVE/DEAD® BacLight™ Viability Kit, Invitrogen/Molecular Probes) were prepared separately as per the manufacturer's instructions. This protocol utilises green-fluorescent SYTO®9 stain to label live bacterial cells green, whereas dead cells are stained red by red-fluorescent propidium iodide. 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.

Organically-grown spinach leaves were aseptically cut into pieces measuring approximately 1 cm by 1 cm and placed onto sterile glass slides. The BacLight stains were added directly to the leaf surface in 250 µl aliquots prior to applying a coverslip on top of the stain. The stained leaf was then incubated in the dark for 30 minutes prior to viewing using Confocal Scanning Laser Microscopy (CSLM). A Leica TCS SP2 UV confocal scanning laser microscope (Leica Microsystems, GMBH, Heidelberg, Germany) was used to observe leaf samples. The samples were scanned with a 488 nm Argon laser for the SYTO®9 stained bacteria using emission wavelengths collected at 500 – 550 nm while a 543 nm Helium/Neon laser for the propidium iodide stained bacteria using emission wavelengths collected at 574 – 714 nm. The microscope was equipped with either X40 HCX Plan (numerical aperture = 0.85) or X63 oil immersion objective (numerical aperture = 1.32) to image the leaf surfaces.

Enumeration of natural flora bacteria on leafy produce after ozone treatment by microscopic analysis and viable (plate) count

Organic baby spinach leaves, Iceberg lettuce, wild rocket, coriander and watercress were aseptically cut into discs using sterile cork borer and placed onto sterile glass slides. According to the ozone sensitivity of these samples (results obtained in year 1), they were treated with 1 ppm ozone concentration, 10 ppm ozone concentration or untreated for 10 minutes. The bacterial staining procedure was performed as mentioned in the previous section. The stains were applied directly to the leaf surface in 250 µl aliquots; a coverslip was placed on top of the stain. Images were captured at 40x magnification. Enumeration was achieved by counting the live cells stained green. Colony counts were also made of each sample using standard plate count agar (PCA) in petri-dishes after serial dilution in minimum recovery diluent (MRD). Incubation of PCA plates was at 30°C for 3 days.

Temperature effects on ozone resistance of P. fluorescens in vitro

Pseudomonas fluorescens was isolated from coriander leaves and then stored at 4°C on Cephaloridin Fucidin Centrimide (CFC) agar plate. To determine the effect of temperature on ozone resistance *in vitro*, a colony of *P. fluorescens* was sub-cultured onto CFC plates and incubated at optimum conditions i.e. 25°C for 48 hrs and/or 4°C to mimic produce storage conditions. A colony of *P. fluorescens* from each temperature plate was added into CFC plates after serial dilution (maintaining temperature conditions) in minimum recovery diluent (MRD). Colony counts were made of each temperature plate using a standard spread plate technique. These plates were then treated with either 1 ppm ozone concentration or ‘clean air’ for 10 minutes. Colony count was determined after incubating CFC plates at 25°C for 2 days.

Age effects on ozone resistance of P. fluorescens in vitro

Pseudomonas fluorescens was isolated from coriander leaves and then stored at 4°C on Cephaloridin Fucidin Centrimide (CFC) agar plate. To determine whether cell age affected the ozone resistance of the bacteria, a colony of *P. fluorescens* was sub-cultured onto CFC plates and incubated at 25°C for varying lengths of time. A single colony was isolated on the 2^{nd}, 4^{th}, 7^{th}, 10^{th} and 12^{th} day of the incubation and transferred to MRD to standardized concentration 10^{-4} cells per mL of each cell age was spread onto sterile CFC plates and these plates were then exposed to either 1 ppm ozone concentration or 'clean air' for 10 minutes.

Ozone resistance of different strains of E. coli: Inoculation of E.coli onto spinach leaves and ozone exposure conditions

E.coli O157:k88a*, E.coli* O25:h4, *E.coli* O128:k67, *E.coli* K12, *E.coli* O55:K59 and *E.coli* O104:h12 were obtained from a culture collection maintained by Geneius Laboratories Ltd. (INEX Business Centre, Newcastle University Campus, Newcastle upon Tyne, UK). Cultures were stored at 4°C on Luria-Bertani (LB) agar plates and were activated in Luria-Bertani (LB) broth at 37°C. The spinach leaves were then aseptically cut into discs using sterile cork borer. A suspension of *E.coli* (overnight culture, 10^8 – 10^9 CFU/ml LB broth) was applied directly to the leaf disc in 300 µl aliquots and then the inoculated leaves stored overnight at 7°C to mimic produce storage conditions and to allow attachment of *E. coli* to the leaf surface. Inoculated leaves were either exposed to 1 ppm ozone or 'clean air' for 10 minutes. The number of *E. coli* remaining (untreated control and ozone exposed) was determined by vigorously shaking the leaf disc in MRD for 2 minutes and then serially diluting in diluent followed by pour plate technique using Tryptone Bile X-Glucuronide (TBX) agar plates. Plates were incubated at 44°C for 24 h, and presumptive colonies were counted.

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

Confocal microscopy: Visualization of bacteria on spinach leaves

Spinach leaves were observed using confocal scanning laser microscopy in conjugation with LIVE/DEAD® BacLight™ Viability Kit (Invitrogen/molecular probes) to see if the bacteria that survived ozone treatment were typically in colonies or individual cells. The large aggregation of live cells stained green is visible in Figure 7 (A) indicated by the blue arrow, at x63 magnification, scale bar = 23.8µm. Lots of individual small colonies and cells in two/threes indicated by the yellow orange arrows (Figure 7A) respectively were observed. The bacteria mainly appeared to be rod shaped. Bacteria in chains indicated by the red arrow were also visible. Individual dead cells stained red are visible in Figure 7 (A) indicated by the white arrow. Figure 7 (B) shows bacteria were attached mainly to the leaf epidermal cell margins, observed at x63 magnification, scale bar = 47.6 µm. Similar bacterial aggregates were also observed on watercress, coriander, rocket and lettuce leaf surfaces (data not shown).

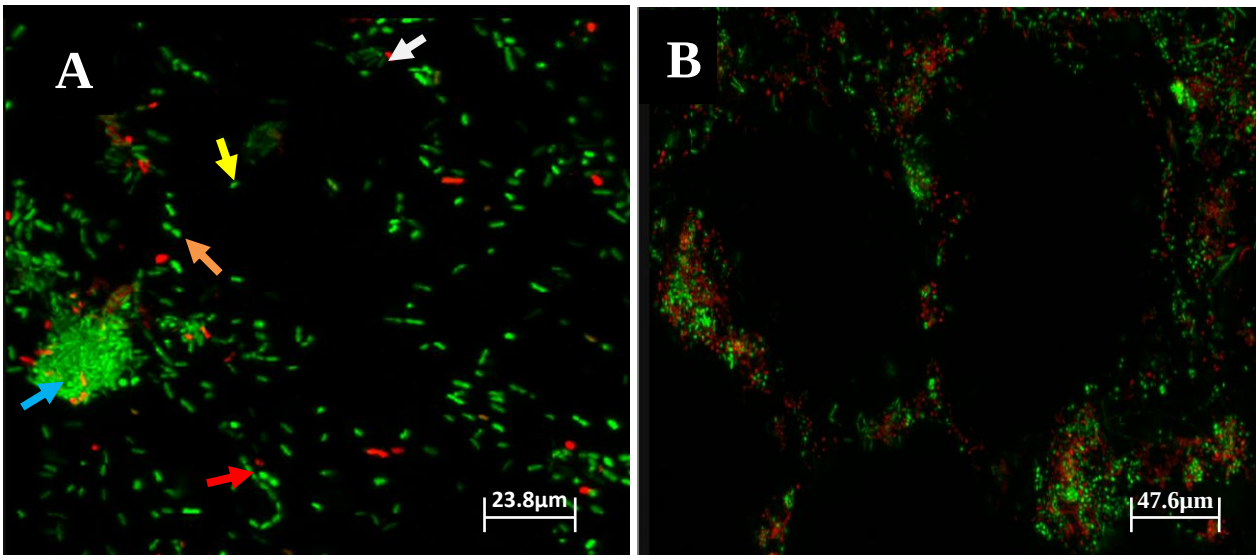

Figure 7: Confocal microscopy image of the baby spinach leaf. (A) Bacteria were stained with green-fluorescent SYTO®9 to label live bacterial cells green and with red-fluorescent propidium iodide to label dead bacterial cells red. Scale bar = 23.8 µm (B) the bacteria appeared to attach preferentially to the epidermal cell margins. Scale bar = 47.6 µm

Bacterial viability on leafy produce after ozone treatment: a comparison of direct microscopic count and indirect plate count methods

Using direct confocal microscopic observation on non-ozone exposed leaves (control), bacteria viability was nearly 90% (Figure 8A, raw data in Appendices), and whereas only 20% of bacteria on an ozone treated leaf surfaces appeared viable. This reduction in bacterial viability was significant (P = 0.001). The yellow arrow (Figure 8B) indicates individual bacteria surviving ozone treatment

while the white arrow (Figure 8B) indicates two/three live cells in a micro-colony of dead cells surviving ozone treatment. Plate counts also confirmed that microbial numbers on spinach leaf were significantly reduced (P = 0.000) by ozone treatment (Figure 8A). Similar results were also observed on watercress, coriander, rocket and lettuce leaf surfaces (Figure 9).

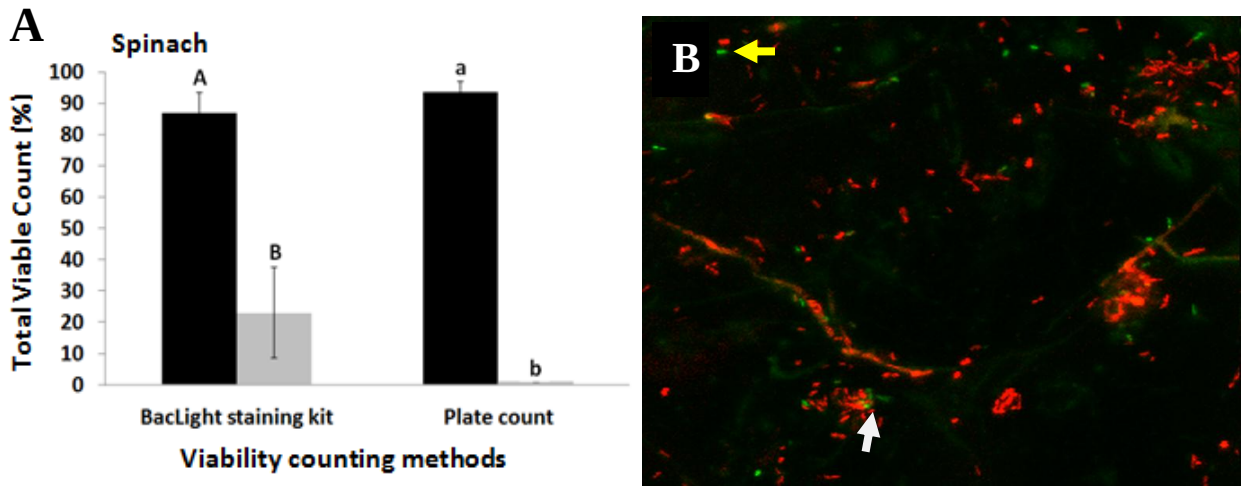

Figure 8: (A) Total viable count (%) of spinach leaves when treated either with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 10 minutes obtained using either direct microscopic counting of SYTO®9/PI stained bacteria on leaves and culture of bacteria on Plate count agar. Values represent means (+/- Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are statistically significantly different (P < 0.05) (B) Confocal microscopy image of the baby spinach leaf when treated with 1 ppm ozone concentration for 10 minutes. 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.

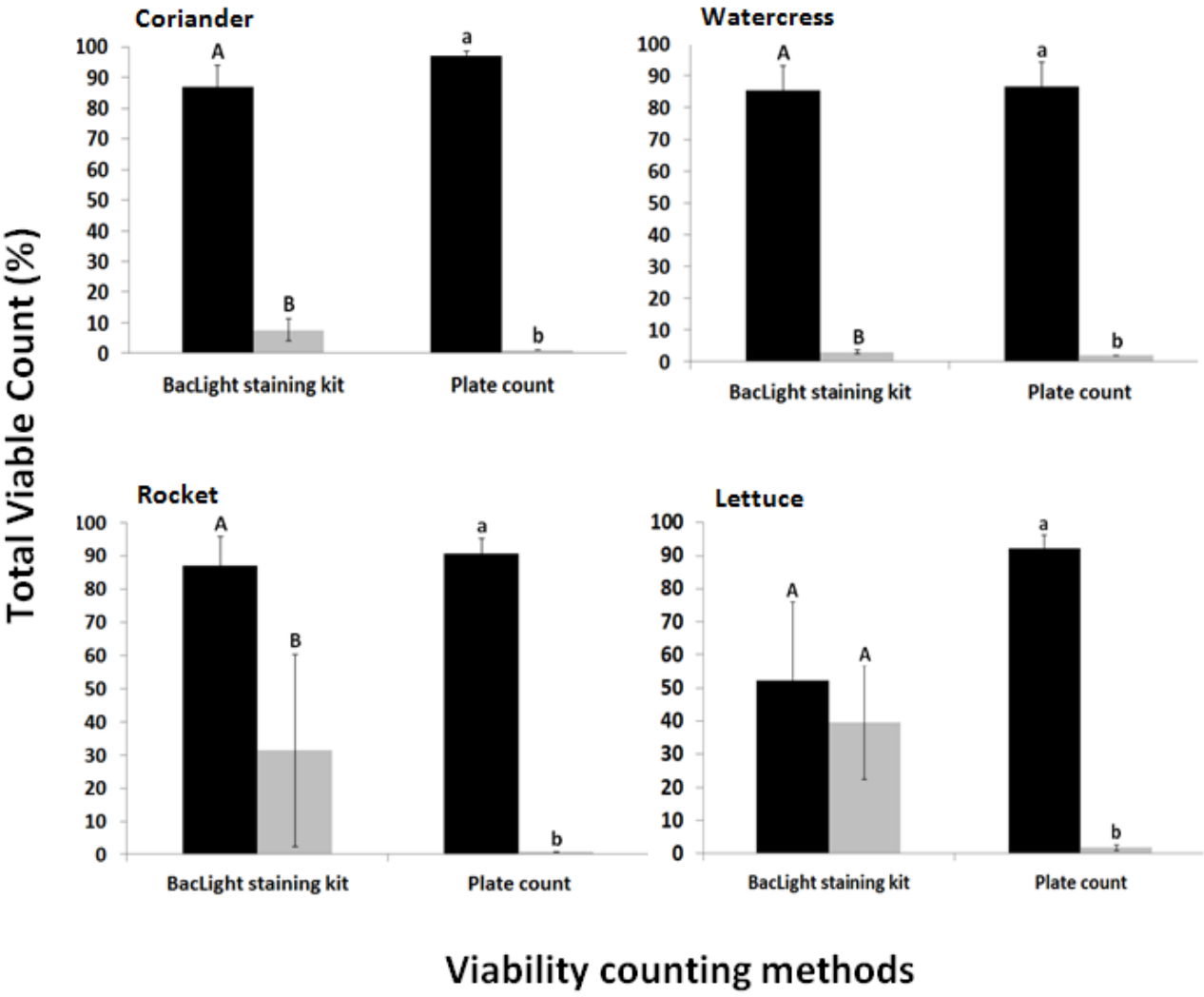

Figure 9: Total viable count (%) of coriander, watercress, rocket & lettuce leaves when treated either with 1 ppm/10ppm ozone concentration (grey bar) or untreated (black bar) for 10 minutes obtained using either direct microscopic counting of SYTO®9/PI stained bacteria on leaves and culture of bacteria on Plate count agar. Values represent means (Standard Error) of measurements made on three independent leaves per treatment. Bars with different letters are statistically significantly different ($P < 0.05$)

Effect of temperature on ozone resistance of P. fluorescens in vitro

Images from confocal microscopy of ozone treated leaves revelaed that two/three live cells survived in micro-colonies surrounded by dead cells and also individual cells survived indicating that these bacteria are ozone resistant. To find potential reasons for this, effect of temperarture (mimicking cold storage conditions) on ozone resistance of *P. fluorescens in vitro* was studied as one stress is known to increase resistance to another stress type.

Colony numbers (CFU) of *Pseudomonas fluorescens* maintained in optimum conditions (25°C) *in vitro* (i.e. where bacteria were grown and exposed to ozone on CFC plates) were significantly (ANOVA, $P < 0.05$) reduced by ozone treatment (Figure 10). In contrast, the colony numbers of *P. fluorescens* maintained in cold conditions (i.e. storage temperature 4°C) *in vitro* were not significantly (ANOVA, $P < 0.05$) reduced by ozone enrichment (Figure 10, raw data in appendices) indicating that cold stress enhances ozone resistance of the leaf surface bacteria.

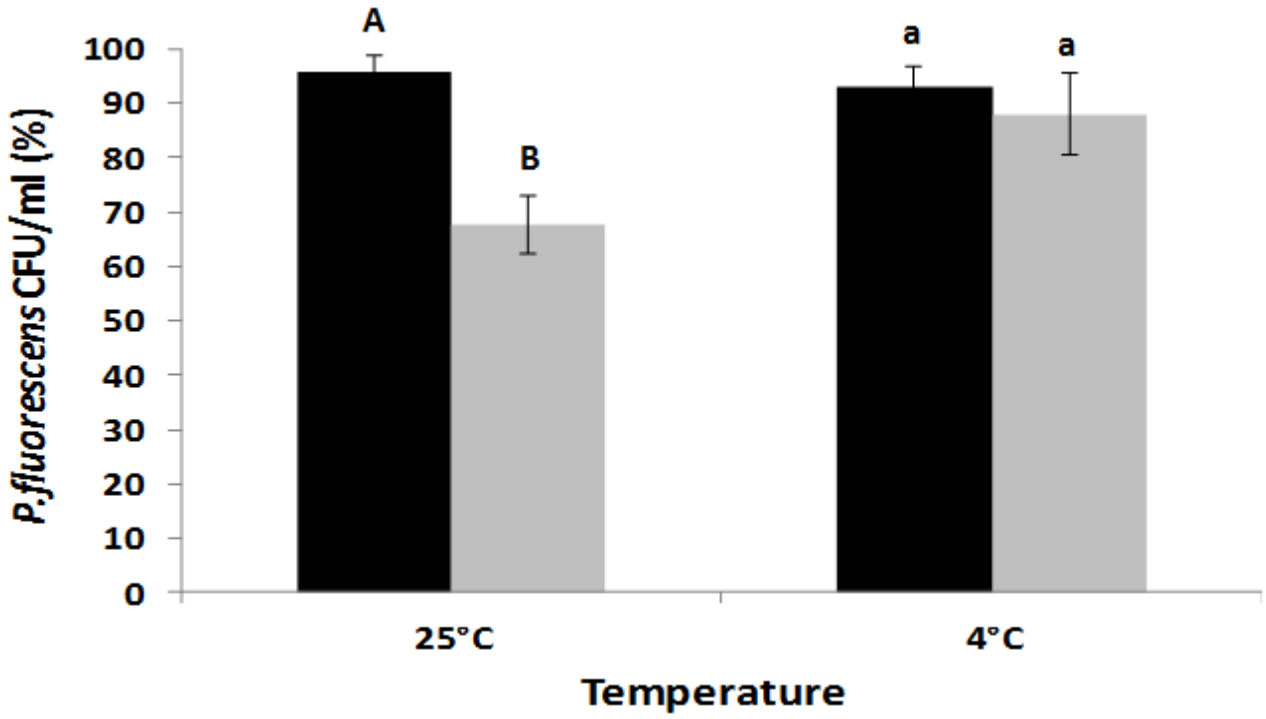

Figure 10: Impacts of ozone-enrichment on *P. fluorescens* grown at 25°C and 4°C and then exposed to either 1 ppm ozone concentration (grey bar) or 'clean' air (black bar) for 10 minutes. After the treatment the plates were either incubated at optimum temperature i.e. 25°C or maintained in cold/storage condition i.e. 4°C. Values represent means (±Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

Effect of age on ozone resistance of the leaf surface bacteria in vitro

After ozone treatment, survival of *P. fluorescens* (*in vitro*) was observed to be greater after 7 days of growth compared to day 2 and day 4 time points and this increased level of survival was maintained at day 10 and day 12 (Figure 11, raw data in appendices) suggesting that older bacteria are more ozone resistant than younger cells.

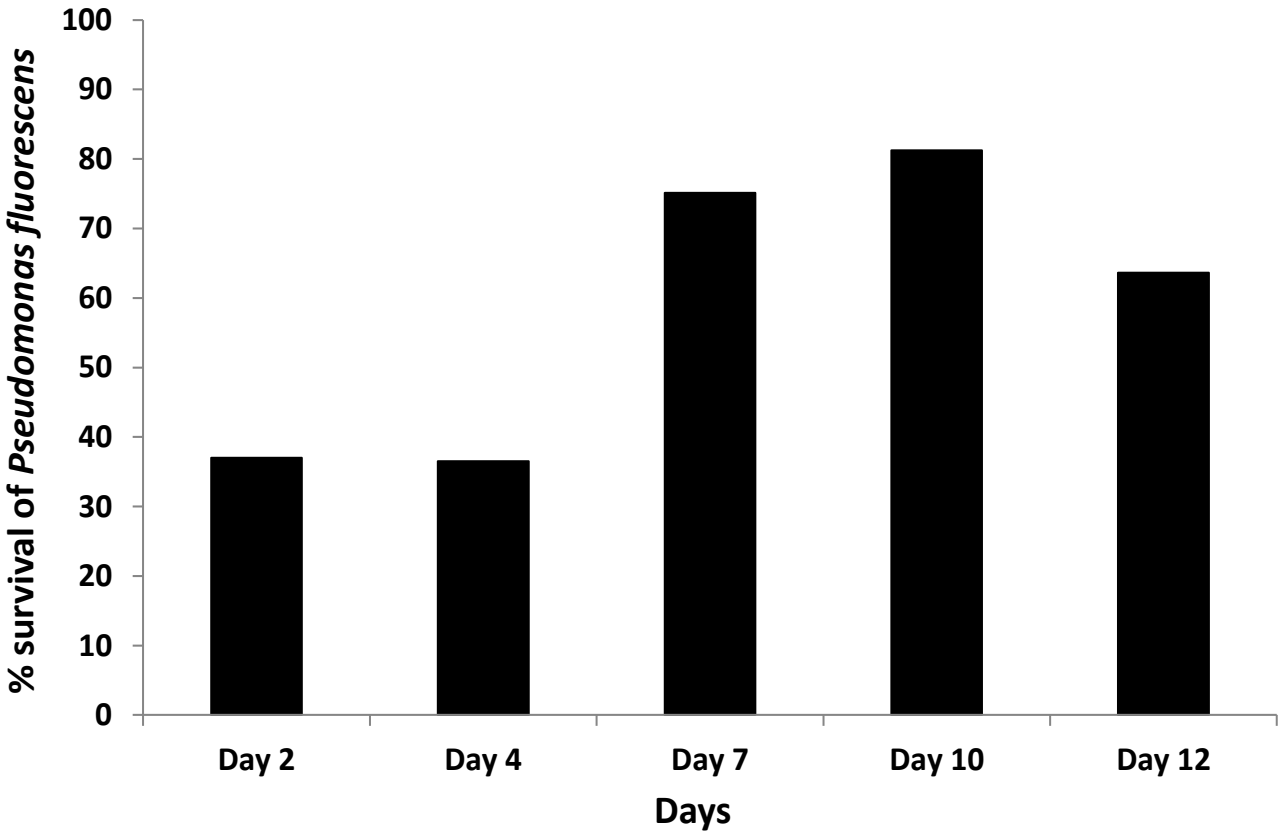

Figure 11: Impacts of ozone-enrichment on *P. fluorescens* exposed 1 ppm ozone concentration for 10 minutes. The culture plate was maintained in optimum temperature i.e. 25°C for 12 days. Values represent means (±Standard Error) of measurements made on three independent plates per treatment.

Effect of ozone exposure on different E. coli strains inoculated onto spinach leaf surfaces

Colony numbers (CFU) of all six strains of *E.coli* i.e. *E.coli* O157:k88a, *E.coli* O25:h4, *E.coli* O128:k67, *E.coli* K12, *E.coli* O55:K59 and *E.coli* O104:h12 obtained from ozone exposed leaves were significantly reduced ($P < 0.05$) compared to non-ozone exposed controls (Figure 12, raw data in appendices). No *E.coli* colonies were isolated from non-inoculated spinach leaves. The bactericidal effect increased with ozone concentration, and length of ozone exposure (results not shown).

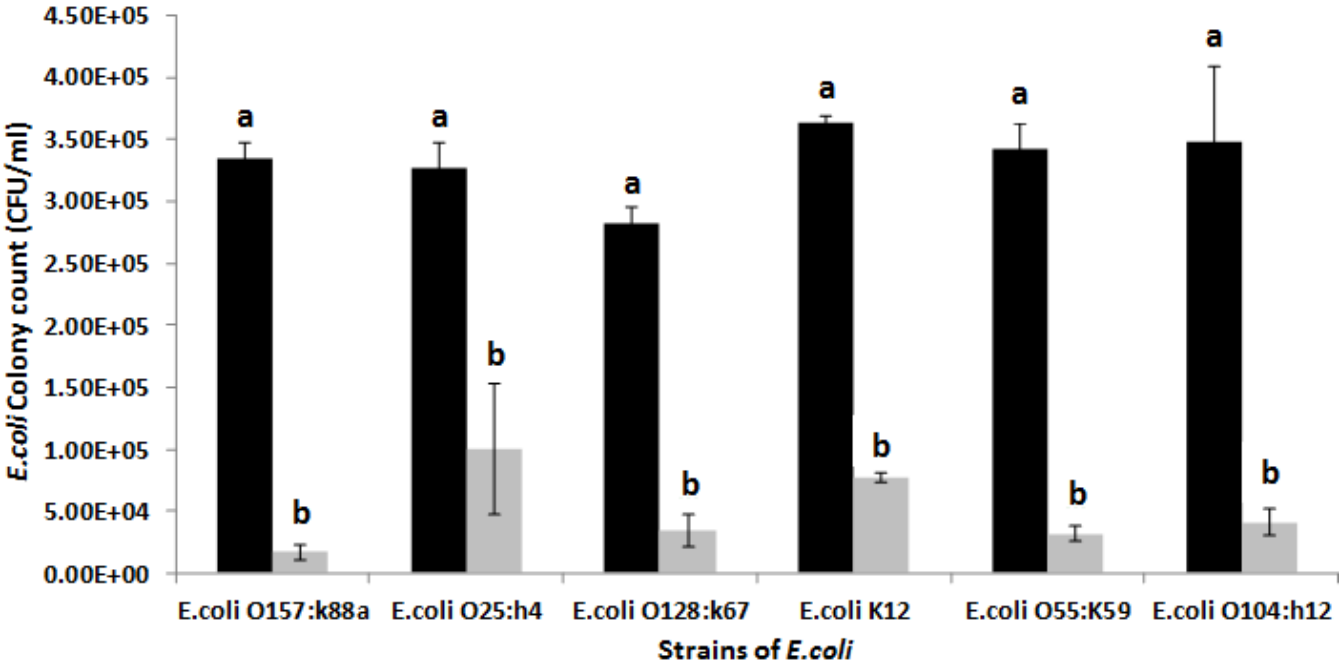

Figure 12: Impacts of ozone-enrichment on six strains of *E.coli* inoculated onto the surface of spinach leaves. Leaves were either treated with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 1 minute. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

Discussion

Confocal microscopy: Visualization of bacteria on spinach leaves:

In this work we observed that bacteria were mainly attached to epidermal plant cell margins and this result is supported by previous authors (Warner *et al*., 2008). SYTO®9/PI staining in conjugation with CSLM allowed *in situ* detection of bacteria on leaf surfaces to be as present in small micro-colonies but mostly as individual cells.

Bacterial viability on leafy produce after ozone treatment: a comparison of direct microscopic count and indirect plate count methods:

The data presented (by direct observation of cells on the plant surface and plate count techniques) indicate that whilst ozone treatment significantly reduced bacterial viable counts on the leaf surface, a number of bacteria were resistant to ozone treatment. Confocal images of ozone treated leaves revealed that two/three live cells survived in micro-colonies of dead cells and few cells on their own indicating lot of variation in bacterial cell death on surface of the leafy produce (see Figure 8B). Individual cells in bacterial micro-colonies may have increased resistance to stress as they will be protected by the exopolysaccharide matrix or by the outermost layer of cells (Mah *et al*., 2001). Individual cells may show ozone resistance for a variety of reasons. For example, Monier and colleagues (2005) observed that a subset of the microbial population is able to survive even under stressful conditions by enhancing their ability to modify the environment. It is also known that pre-exposure to stress can induce resistance to other types of stress (Johnson, 2008) and that age of cells can influence cell survival to adverse conditions (Vulic *et al*., 2001). Overall the results indicate that although the vast majority of bacteria are killed by ozone there are a number of cells that survive and this could be due to a combination of physical protection in a micro-colony and increased stress resistance in certain cells due to environmental conditions and cell age.

Effect of temperature and age on ozone resistance of leaf surface bacteria:

Cold stress: The results indicate that *P. fluorescens* maintained in cold conditions were more resistant to ozone treatment which demonstrates that cold stress enhances ozone resistance of leaf surface bacteria *in vitro*. It is known that stress resistance mechanisms in bacteria are linked and a bacterium exposed to cold stress will also be resistant to other applied stresses. Therefore it is possible that the stresses e.g. cold, drought, applied to bacteria while on the leaf surface actually enhance ozone resistance (Johnson, 2008). Survival of these bacteria in stressed conditions is a combination of cell responses designed to minimise lethal effects or repairing damage (Jozefczuk *et al*., 2010). In case of repairing damage, the presence of cold shock proteins in bacteria overcomes growth limiting effects by either altering redox status or increasing stability of RNA and DNA secondary structures (Reva *et al*., 2006). Cold shock acclimation proteins are exhibited in high abundance during low temperature and have been identified in *Pseudomonas* spp. which may be responsible for the psychrotropic behaviour of the bacteria (Reva *et al*., 2006). Temperature acclimation results from changes in both biochemical and physiological process (Kaplan *et al*., 2004).

Cell age: The data shows that survival of *P. fluorescens* after ozone treatment was affected by cell age with older cells (7-12 days old) being more ozone resistant than younger cells (4 days old). This result is supported by Zambrano and colleagues (1993), who observed increased survival of 10-

day-old culture as compared to 1-day-old culture of the same strain of *E.coli*. This is probably due to bacteria experiencing growth advantage in stationary phase (GASP) phenomenon in which aged cells out-compete younger cells (Finkel, 2006). The bacteria in stationary phase cease the growth of their cells and increases resistances to many environmental stresses by significantly reducing its metabolic activity (Vulic *et al*., 2001).

Therefore, metabolomic and transcriptomic approaches will be designed for further investigation on understanding the stress response in *P. fluorescens*. A better understanding of these resistance mechanisms may lead to future treatments able to overcome them.

Effect of ozone exposure on different E. coli strains inoculated onto spinach leaf surfaces:

The results indicate that 1 ppm ozone concentration for 1 minute can significantly reduce *E.coli* O157:k88a survival. The treatment was also effective on *E.coli* O25:h4 and *E.coli* O128:*k67* confirming that *E.coli* species are sensitive to ozone. Yuk and colleagues (2007) have shown 1 $\log_{10}$ reductions of *E.coli* O157:H7 on mushrooms by ozone treatment up to 3 ppm. Their studies also revealed that *E.coli* O157:H7 to be more sensitive than *Listeria monocytogenes*. The difference in ozone sensitivity may be due to the cell envelope which is possibly the principal target of ozone activity (Yuk *et al*., 2007).

Conclusions

- Confocal scanning microscopy demonstrated that bacterial cells occurred both as micro-colonies and as individuals on the leaf surface
- Cells on a leaf surface appear to survive ozone treatment by a combination of physical protection (micro-colony) and physiological adaptation.
- Cold stress and increasing age of the leaf surface bacteria (*P. fluorescens*) enhances ozone resistance
- *E. coli* are sensitive to ozone treatment

Knowledge and Technology Transfer

‘Best speaker’ award in the Post-graduate Research (PGR) Conference 2013, Newcastle University

Second prize in the NIRes poster competition 2012, Newcastle University

An article of my work was published in ‘Horticulture Week’ magazine in November 2012, in ‘HDC News’ magazine in March 2013 and in ‘Field vegetables’ review 2013 magazine in September 2013

Presented posters in ‘British Leafy Salad Association’ (BLSA) Conference 2012, NIRes poster competition 2012 and Society of General Microbiology (SGM) Conference 2013

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Appendices

Raw data for bacterial viability on leafy produce after ozone treatment: a comparison of direct microscopic count and indirect plate count methods

Spinach (Bacterial count cfu/cm^2)	Viability count using staining			Plate count		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	86	76	99	92	89	100
1 PPM	52	10	7	1	1	1

Coriander (Bacterial count cfu/cm^2)	Viability count using staining			Plate count		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	85	100	76	100	94	97
10 PPM	14	7	2	1	1	1

Watercress (Bacterial count cfu/cm^2)	Viability count using staining			Plate count		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	82	100	74	100	74	86
1 PPM	2	4	3	2	2	2

Rocket (Bacterial count cfu/cm^2)	Viability count using staining			Plate count		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	100	91	70	87	100	85
10 PPM	89	3	2	0.9	0.6	0.8

Lettuce (Bacterial count cfu/cm^2)	Viability count using staining			Plate count		
	Replicate I	Replicate II	Replicate III	Replicate I	Replicate II	Replicate III
Control	26	31	100	90	100	87
1 PPM	74	21	24	2	3	0.3

Raw data for effect of temperature on ozone resistance of leaf surface bacteria *in vitro*

Pseudomonas	**Temperature 25°C**		
	Replicate I	**Replicate II**	**Replicate III**
Control	27000000	25000000	27700000
Into %	97	90	100
1 PPM	19200000	15900000	21100000
Into %	69	57	76

Pseudomonas	**Temperature 4°C**		
	Replicate I	**Replicate II**	**Replicate III**
Control	25000000	23600000	27200000
Into %	92	87	100
1 PPM	26800000	20000000	25000000
Into %	99	74	92

Raw data effect of age on ozone resistance of the leaf surface bacteria *in vitro*

***Pseudomonas* at 30°C**	**control 1**	**control 2**	**control 3**	**ozone 1**	**ozone 2**	**ozone 3**
Day 2	13100000	14300000	12600000	5000000	4500000	5300000
Day 4	8400000	8900000	9000000	3200000	3000000	3400000
Day 7	20600000	15100000	17000000	14000000	14000000	11600000
Day 10	7900000	7000000	6400000	5000000	6400000	5900000
Day 12	1000000	400000	800000	600000	300000	500000

Raw data for effect of ozone exposure on different *E. coli* strains inoculated onto spinach leaf surfaces

***E.coli* spp.**	**Replicate no.**	**Control - No ozone treatment**	**1 ppm ozone treatment**
***E.coli* O157:k88a**	**I**	356000	19000
	II	312000	6000
	III	335000	26000

***E.coli* O25:h4**	**I**	364000	204000
	II	320000	28000
	III	297000	69000
***E.coli* O128:k67**	**I**	260000	10000
	II	302000	41000
	III	287000	52000
***E.coli* K12**	**I**	375000	85000
	II	353000	76000
	III	361000	72000
***E.coli* O55:k59**	**I**	306000	36000
	II	340000	20000
	III	378000	40000
***E.coli* O104:h12**	**I**	416000	42000
	II	400000	59000
	III	229000	22000