Grower Summary

FV 386

Use of gaseous ozone to prevent microbial postharvest spoilage and reduce pesticide residue levels

Annual 2013

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Only officially approved pesticides may be used in the UK. Approvals are normally granted only in relation to individual products and for specified uses. It is an offence to use non-approved products or to use approved products in a manner that does not comply with the statutory conditions of use, except where the crop or situation is the subject of an off-label extension of use.

Before using all pesticides check the approval status and conditions of use.

Read the label before use: use pesticides safely.

Further information

If you would like a copy of the full report, please email the HDC office (hdc@hdc.ahdb.org.uk), quoting your HDC number, alternatively contact the HDC at the address below.

HDC
Stoneleigh Park
Kenilworth
Warwickshire
CV8 2TL

Tel – 0247 669 2051

HDC is a division of the Agriculture and Horticulture Development Board.

Project Number:	FV 386
Project Title:	Use of gaseous ozone to prevent microbial postharvest spoilage and reduce pesticide residue levels
Project Leader:	Dr Ian Singleton; Shreya Wani (Student)
Contractor:	Newcastle University
Industry Representative:	Thane Goodrich, Intercrop Ltd and Steve Rothwell, Vitacress Salads Ltd
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Start Date:	01 October 2011
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Project Cost:	£83,150

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).

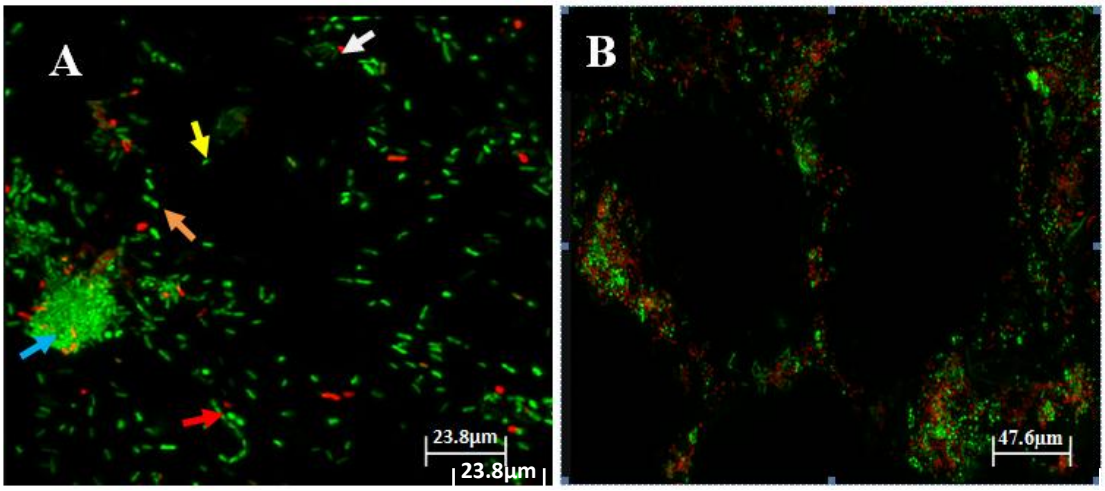

Figure 1: Confocal microscopy image of a baby spinach leaf. (A) Bacteria were stained with green-fluorescent SYTO®9 to label live bacterial cells green and with red-fluorescent propidium iodide to label dead bacterial cells red. Scale bar = 23.8 µm (B) the bacteria appeared to attach preferentially to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

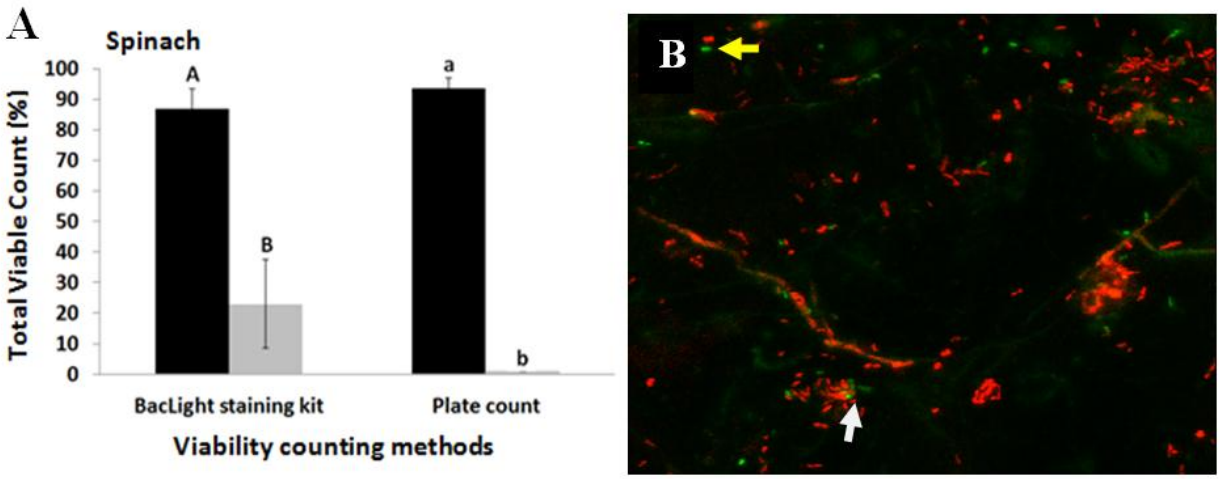

Figure 2: (A) Total viable count (%) of spinach leaves when treated either with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 10 minutes obtained using either direct microscopic counting of SYTO®9/PI stained bacteria on leaves and culture of bacteria on Plate count agar. Values represent 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.

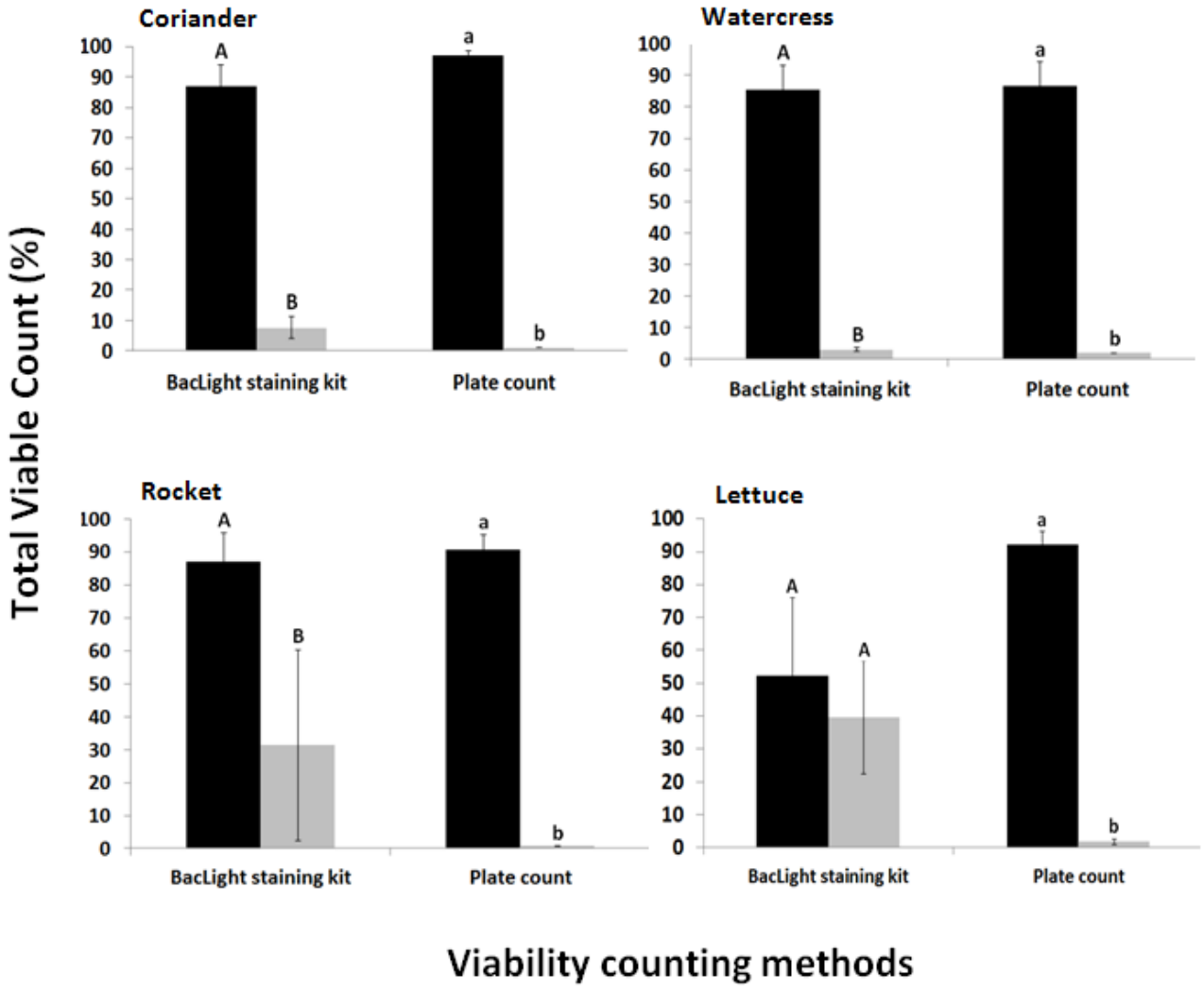

Figure 3: Total viable count (%) of coriander, watercress, rocket & lettuce leaves when treated either with 1 ppm/10ppm ozone concentration (grey bar) or untreated (black bar) for 10 minutes obtained using either direct microscopic counting of SYTO®9/PI stained bacteria on leaves and culture of bacteria 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.

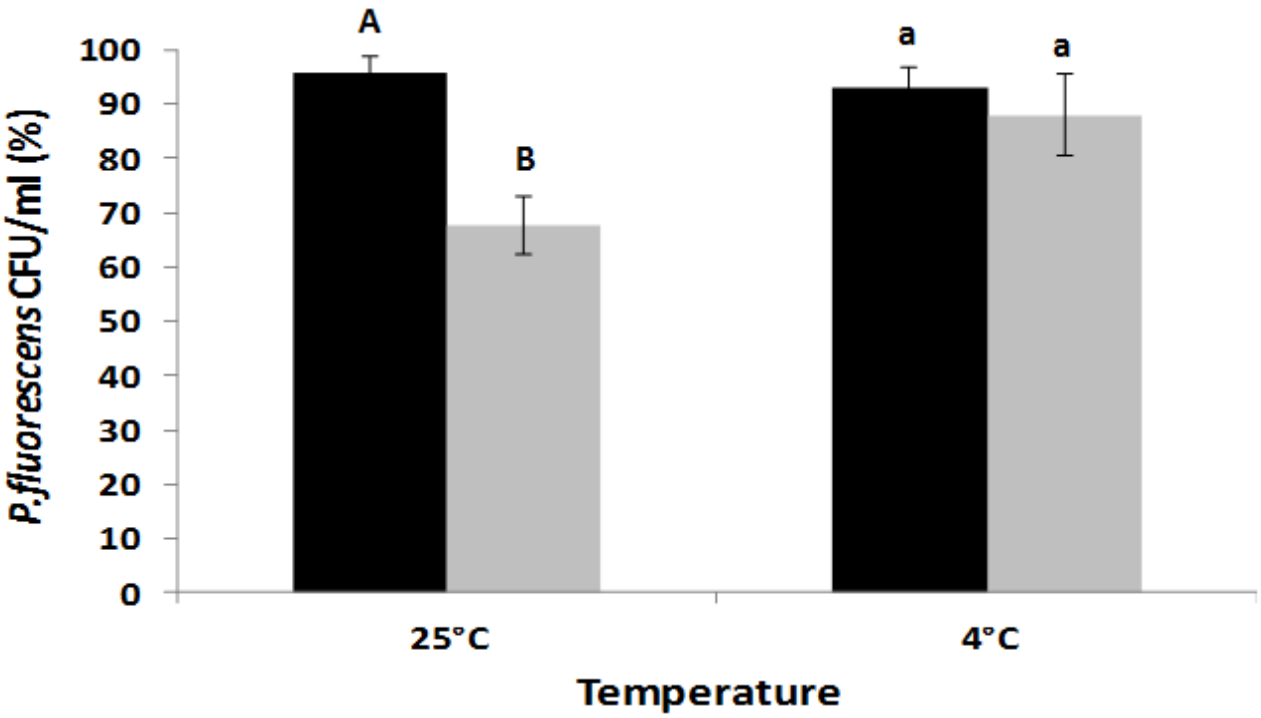

Figure 4: Impacts of ozone-enrichment on *P. fluorescens* grown at 25°C and 4°C and then exposed to either 1 ppm ozone concentration (grey bar) or 'clean' air (black bar) for 10 minutes. After treatment the plates were either incubated at optimum temperature i.e. 25°C or maintained in cold storage conditions 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.

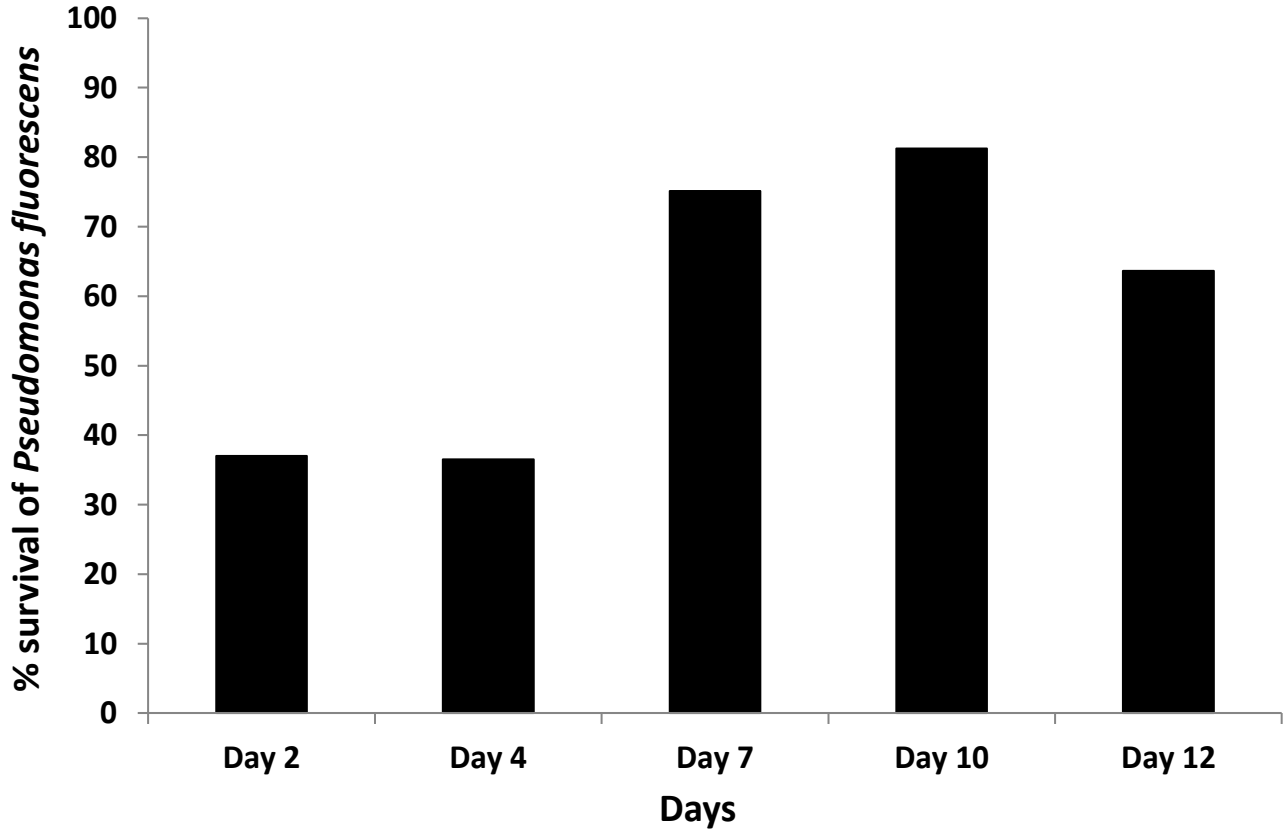

Figure 5: Impacts of ozone-enrichment on *P. fluorescens* exposed 1 ppm ozone concentration for 10 minutes. Culture plates were maintained in optimum temperature i.e. 25°C for 12 days. Values represent means (±Standard Error) of measurements made on three independent plates per treatment.

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).

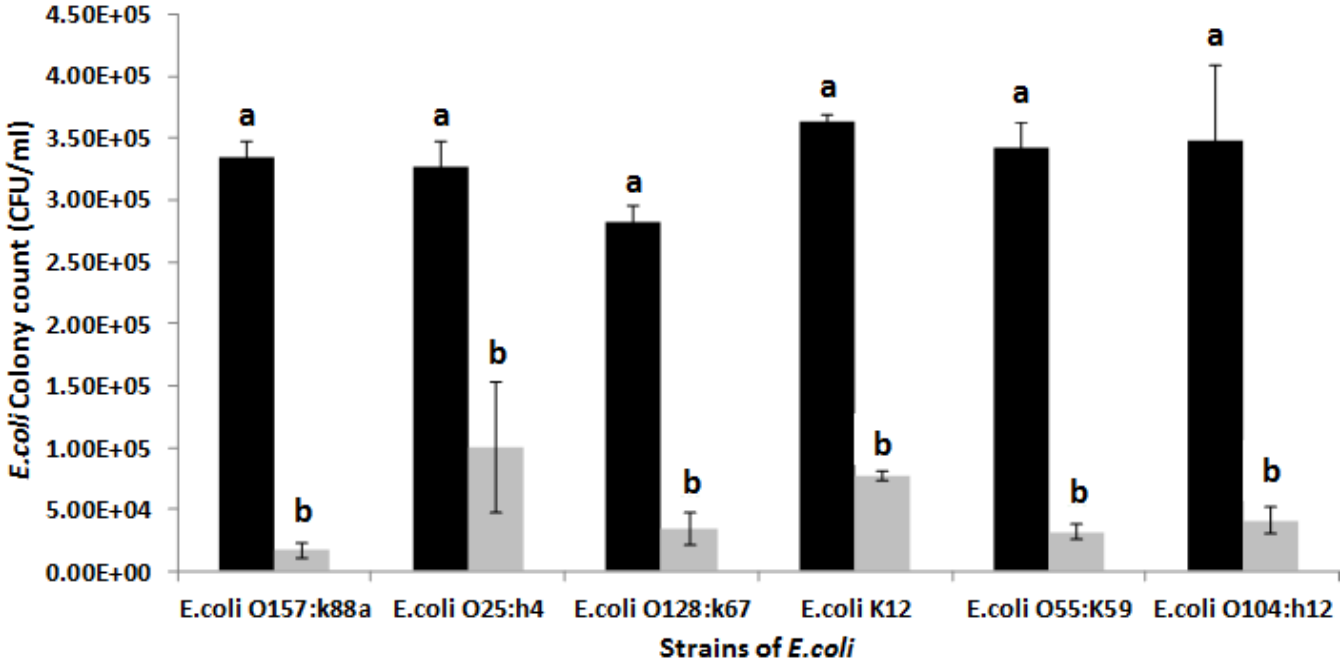

Figure 6: Impacts of ozone-enrichment on six strains of *E.coli* inoculated onto the surface of spinach leaves. Leaves were either treated with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 1 minute. Values represent means (Standard Error) of measurements made on three independent 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.