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Location of project:	Lancaster Environment Centre
Industry representative:	David Norman, FPC
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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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GROWER SUMMARY

Headline

The application of the rhizobacteria *HDC P003* to lettuce plants in propagation or in the field increased lettuce head weight by up to 19% in 4 out of 6 cultivars. However, the development of this organism as a commercial product is at the "proof of concept" stage.

Background and Expected Deliverables

In the UK, lettuce comprises the majority of the salad leaf industry worth £566 million a year, with lettuce being bought by 92% of consumers (Ceres 2012). Over recent years, there has been increasing demand for more cultivars, with salad bags and bowls often containing a mixture of varieties. Due to this high demand, lettuce needs to be grown all year around (either abroad or in greenhouses during winter) and thus may be exposed to environmental stresses that limit productivity.

Environmental stresses such as drought stimulate the production of the growth inhibitory phytohormone ethylene (Belimov *et al.*, 2009). There is considerable horticultural interest in decreasing the production of ethylene, or antagonising its effects. One method to achieve this is soil inoculation with rhizobacteria containing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCd), which hydrolyses the precursor of ethylene, ACC, into α-ketobutyrate and ammonia (Honma and Shimomura 1978). Previous work (within HDC Studentship CP 54) has shown that rhizobacteria that modify crop hormone status (*V. paradoxus* 5C-2, *Bacillus subtilis* IR-15) increased lettuce head weight of crops grown under both well-watered and drying soil conditions in both pot experiments and small-scale (6 m^2 cropping area per treatment) polytunnel experiments.

This project explored the use of *HDC P003* (a very similar ACCd-containing rhizobacterium) as an inoculant for lettuce grown under commercial cropping conditions. Suitable propagation and inoculation techniques for large-scale inoculum production using both commercially available (*Bacillus subtilis* QST713, marketed as Serenade ASO by Bayer Crop Science) and experimental inoculants (available at Lancaster University) were determined. Consequently, the deliverables of the project are:

- the development of suitable inoculation techniques for several different rhizobacterial inocula intended for field lettuce production
- the documentation of data showing the effects of two different rhizobacterial inoculants on rhizobacterial colonisation of the root zone, and the incidence of plant disease and crop

productivity

Summary of the project and main conclusions

Field trials were conducted using the summer outdoor lettuce cultivars Iceberg, Endive, Red Salanova and Red Coral (August-October) and winter indoor lettuce Green Oak Leaf (Cook) and Apollo (Exact) (November-January) at different sites with plants grown in field soil. When applied according to the manufacturer's instructions, the commercially available product Serenade (*Bacillus subtilis* QST713) had no effect on lettuce head weight (presumably since disease incidence in the crop was low). When applied at the same bacterial concentration, *HDC P003* increased yield by up to 19% according to site and cultivar (in 4 of 6 cultivars tested). Adding rhizobacteria directly to plants in the field generally resulted in more consistent effects on crop yield than inoculation in the nursery.

Although variability in crop response to PGPR may be caused by variation in rhizobacterial colonisation of the root system, there is currently no method to quantify *HDC P003* colonisation of the root system. Consequently, molecular work aimed to provide a sensitive DNA-based technique by sequencing the 16S gene of *HDC P003.* However, there was no difference in probe detection when root systems were recovered from inoculated or uninoculated plants. Greater primer specificity needs to be achieved, and work (still ongoing) aims to achieve this, by using the ACCd gene.

Greenhouse-based pot trials conducted during winter aimed to select appropriate inocula and inoculation techniques for field experiments. *HDC P003* increased head weight, leaf area and water use efficiency of iceberg lettuce grown in soil from the field, similar to its effects in field trials.

Financial Benefits

Assuming a 20% increase in lettuce head weight, and a wholesale value of 33 pence per head for butterhead lettuce (Gov.uk 2014) or Little Gem (Horticulture Week 2014) lettuce, this would represent additional earnings if price was based on weight. Alternatively, should the crop achieve the desired minimum harvestable head weight 2 weeks earlier, this may allow an additional crop in each season.

Action points for growers

To note that *HDC P003* can be applied during propagation or post-planting and stimulates lettuce growth. However, it should be stressed that development of this organism as a commercial inoculant is at “proof of concept” stage, and there is currently no commercially available product.

SCIENCE SECTION

Introduction

Many commercially available inoculants exploit the ability of rhizobacteria to improve crop yield by acting as biofertilisers or biopesticides. Biagro (Glenside, Livingston, UK) contains a mixture of naturally occurring soil microorganisms and can be applied to lettuce. The majority of biopesticides inhibit fungal diseases (Nakkeereen *et al*., 2006), thereby minimising yield losses. Examples of biopesticides which include lettuce as a target crop are: Contans (Sipcam Agro, USA) (Chitrampalam *et al*., 2011), Plantshield (BioWork, USA), Soilgard (Certis, USA), Salavida (Sourcon Padena, Germany) (Berg 2009), Companion (Growth Products, USA), Sonata (Bayer CropScience, Germany) (Cao *et al*., 2010) and Serenade (Bayer CropScience, Germany) (Berg 2009). The active agent of most are either fungal or *Bacillus spp.*; *B.subtilis OST 713* in the case of Serenade. The common soil bacterium *Variovorax paradoxus* (Willems *et al*., 1991) suppressed plant fungal disease (Berg *et al*., 2005) but is not commercially available. Preliminary experiments suggest that *V. paradoxus 5C-2* decreased the number of culturable fungi on lettuce roots (Teijeiro 2012), but its effects on plant disease have not been compared with other products.

This project aimed to further develop a promising experimental inoculant selected as a growth stimulant, especially when plants are exposed to environmental stresses. *V. paradoxus 5C-2* stimulated vegetative growth of diverse species such as *Arabidopsis* (Chen *et al*. 2013), peas (Belimov *et al*., 2009; Jiang *et al*. 2012) and lettuce (Teijeiro *et al*. 2011) grown under well watered or drought conditions. Environmental stresses stimulate ethylene production, a potent plant growth inhibitor. *V. paradoxus 5C-2* contains the enzyme ACC deaminase (ACCd) which metabolises the precursor of the plant hormone ethylene, thereby decreasing plant ethylene synthesis and increasing growth. Among the many strains of *V. paradoxus* that contain ACCd, *HDC P003* was chosen for this project since previous work demonstrated it had the greatest effect on lettuce root growth *in vitro* (Teijeiro *et al*., 2011).

This project developed field trials to test the effects of *HDC P003* as a potential growth stimulant on field- and glasshouse-grown lettuce crops, in collaboration with two commercial

growers in different parts of the country. Three (of 4) trials compared *HDC P003* with the commercial inoculant Serenade and the effects of adding growth media alone (without live bacterial cells). Root samples were collected from lettuce plants to assay microbial colonisation via real time PCR. Pot trials compared the application methods (dosage and media) used in field trials. This work supports endeavours to develop *HDC P003* as a commercial product to stimulate growth and alleviate effects of environmental stresses such as drought. However, it should be stressed that development of this organism as a commercial inoculant is at "proof of concept" stage, and there is currently no commercially available product.

Materials and methods

Culturing of HDC P003 for field trials

HDC P003 was stored at -80°C in Bacto Pseudomonas F (BPF) broth plus 15% glycerol. From storage, it was streaked onto tryptic soy agar and either incubated on the bench for up to 5 days or grown over 2 days at 30°C. It was checked for purity by gram stain and colony type. Both *HDC P003* and *5C-2* have distinctive small gram negative cells which do not form pairs or chains, unlike some other members of the *Variovorax* genus (Willems *et al*., 1991). *HDC P003* produces one colony type which is light yellow (Willems *et al*., 1991), with umbonate elevation and an undulate margin. Once purity of the strain was ascertained, tryptic soy broth (TSB) was inoculated with *HDC P003* and incubated on a shaker over night at 28°C and 250 rpm in conical flasks. For all experiments, the optical density at 600 nm (OD_{600}) of the broth cultures was 2 (10^{10} colony forming units, CFU mL^{-1}). Broth was transferred to sterile bottles for transport to the field.

Experiment 1 (Field Trial)

Inoculation with *HDC P003* (in TSB) was compared against an untreated control using the cultivar Iceberg. One litre of bacterial culture was diluted on site to 1/10 (10^9 CFU mL^{-1}) with unsterile tap water and applied to iceberg seedlings in seedling trays (peat blocks) via a watering can. One litre of inoculant was applied to 150 seedlings at the 2 leaf stage. Control plants received an equivalent volume of tap water. The plants were transplanted to the field a week later (Fig. 1) at a plant density of 7 plants m^{-2}. A furrow (66 cm) separated inoculated plots from control plots. Each treatment comprised 200 plants (100 per block). After two months when plants were grown under commercial conditions, the lettuces were harvested in a randomised order and head weight recorded.

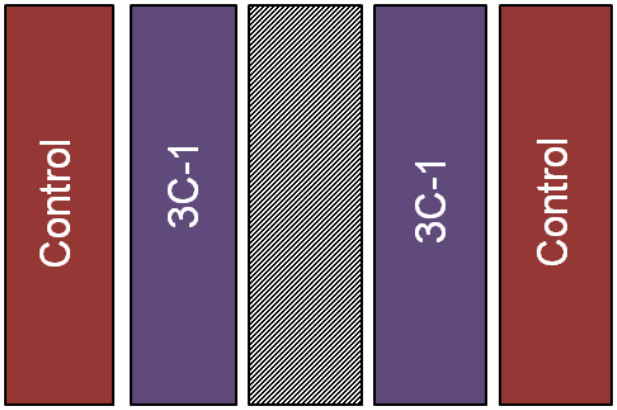


Figure 1: Field layout of Iceberg in Experiment 1.

Experiment 2 (Field Trial)

Inoculation with *HDC P003* (in TSB) was compared with the commercial product Serenade (Bayer CropScience). Two additional controls (TSB only and sterilised Serenade) checked for effect of bacterial growth medium. Serenade was sterilised by heating at 120°C-134°C for 20 minutes to inactivate the *Bacillus*. Unlike the previous trial, all treatments were applied using pressurised sprayers directly onto the plants in the field. The cultivars treated were: Endive (transplanted on the day of bacterial inoculation), Red Coral and Red Salanova (both transplanted the day before inoculation). The experimental layout is given in Figure 2. To ensure a fair comparison of the two bacterial inoculants, the concentration of *HDC P003* was kept the same as that of Serenade, which was applied according to the manufacturer's instructions: 1 mL in 40 mL water per m^2 (10^8 CFU mL^{-1}). Planting density for Red Salanova, Red Coral and Endive was 20, 16 and 12 plants m^{-2} respectively, thus each plant received 0.5, 0.7 and 0.9 mL of inoculum respectively. As with Experiment 1, each plot consisted of a single treatment and a buffer zone of at least 0.66 m separated different treatments to prevent cross-contamination. Plants were grown in the field for two months, and then harvested in randomised blocks of 6 plants for Endive, 15 for Red Coral and 18 for Red Salanova, for which 5 trimmed heads per block were weighed.

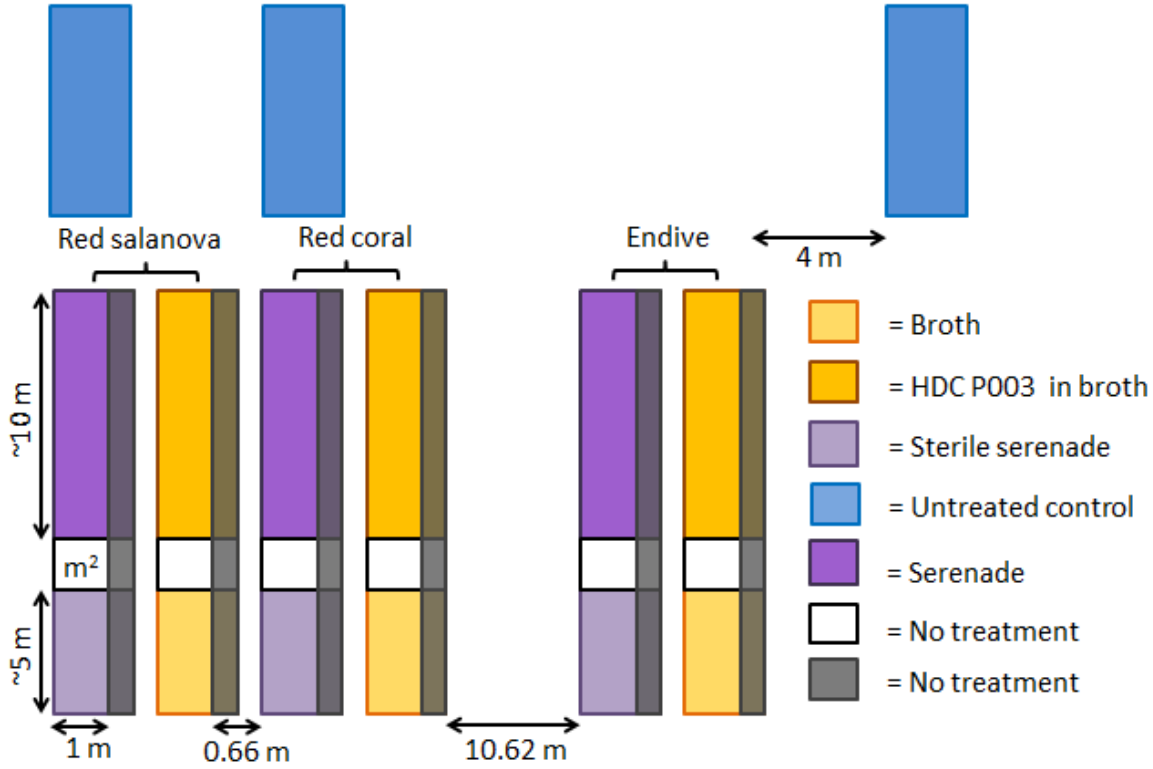
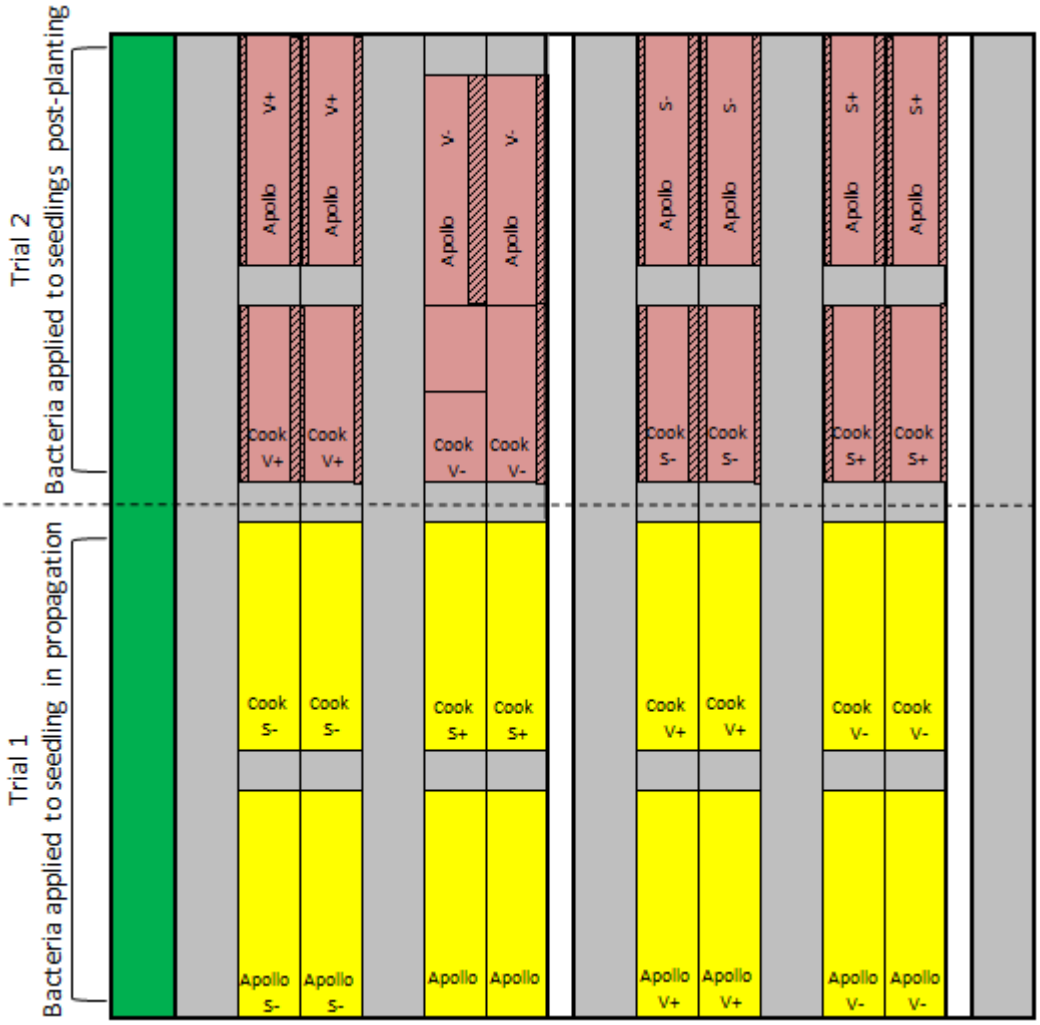


Figure 2: Field layout of Experiment 2.

Experiment 3 (Protected Crop)

Experiment 3 utilised 2 different methods of inoculant application: bacteria applied to seedlings during propagation (as in Experiment 1) and bacteria applied to seedlings post-planting (as in Experiment 2), which was set up a week later. The same 5 treatments were applied as in Experiment 2 with one modification. The corresponding control for *HDC P003* applied sterilised HDC P003 in broth (rather than TSB broth alone) in case bacterial exudates (during storage of inoculum) affected plant growth. Control treatments of both *HDC P003* and Serenade were sterilised by heating at 120°C-134°C for 20 minutes. When live cultures were applied to plants, an OD_{600} of approximately 2 was required as a stock solution. When bacteria was applied to seedlings, treatments were diluted 1/10 (OD_{600} of 0.2) and 385 mL applied to a tray comprising 150 seedlings in peat blocks using a watering can. Each treatment was applied to two trays. These were transplanted 4 days later to a greenhouse.

One week later, the same 5 treatments were applied directly to lettuces that had already been transplanted into the glasshouse. The treatments were diluted to 1/20, which supplied 10^8-10^9 CFU mL^{-1} for both inoculants. Plants on the plot edges, which would not be harvested to determine head weights, were not inoculated to concentrate the inoculum within the plots. For each trial, two cultivars, Cook and Apollo, were tested. The experimental layout is given in Figure 3. Untreated Apollo were planted as buffers between the treatments and used as controls for that cultivar. All plants were not treated with any other biological growth stimulant prior to inoculation with *HDC P003* or Serenade, aside for the untreated controls of Cook, to which Biagro (Glenside, UK) was applied, as in commercial practice. Contractor harvesting of Apollo (when bacteria were applied to seedlings post-planting*)* prior to research staff availability meant that insufficient plants were available for proper replication. Individual head weights were taken of each lettuce from 6 groups of 15 per treatment in a randomised order for Cook (Fig. 4) and 20 per treatment for Apollo seedlings treated with rhizobacteria in propagation. Plant density was ~4 per m^2.



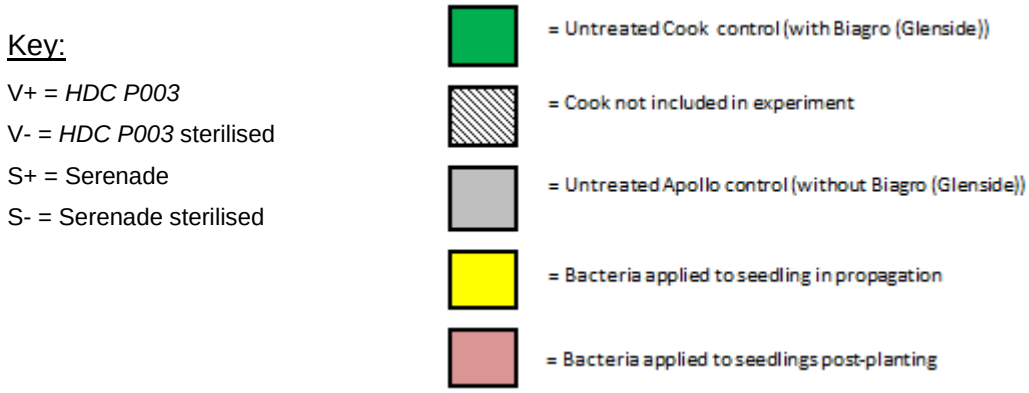


Figure 3: Field Layout of Experiment 3

Figure 4: Harvest of Experiment 3.

Experiment 4 (Protected Crop)

Inoculation with *HDC P003* (in TSB) was compared with the commercial product Serenade (Bayer CropScience), with 2 irrigation treatments overlaid. Lettuce (*Lactuca sativa) 'Frank'* seed was sown on 22 July 2013, and seedlings received two pesticide applications prior to planting: Fubol Gold (fungicide), Switch (fungicide) and Aphox (insecticide) on 29 July 2013 and Amistar (fungicide) Inca on 2 August 2013. Seedlings were transplanted on 15 August 2013 to a polytunnel at Myerscough College (Bilsborrow, Preston) into 4 beds which were fully irrigated for one week following transplanting. Thereafter, two treatments were applied such that two beds received no further irrigation while two additional beds were irrigated every day for 20 minutes if soil moisture fell below a pre-set threshold (determined from

previous work). Soil moisture sensors (SM200, Delta-T Devices, Burwell, UK) could activate irrigation if θ decreased below 30% v/v. On the day of transplanting, 3 treatments (Serenade, *HDC P003*, and a water control) were applied. Inoculation comprised 1 mL of concentrate in 40 mL of water. Planting density was 20 plants m^{-2}; therefore, each plant had 2 ml of solution directly applied. Treatments were arranged in 1 m^{-2} plots with 0.75 m between plots to avoid cross contamination (Fig. 5). Despite rabbit fencing surrounding the polytunnel, some damage to plants was apparent three weeks after transplanting, but all treatments were equally affected. Irrigation treatments continued for a further eight weeks before lettuce plants were harvested on 16 October 2013. At this time, fresh and dry weight were recorded. Furthermore, volumetric soil water content (θ) was recorded in each plot at two depths (0-10 cm and 15-25 cm) using a theta probe (Delat-T devices, Burwell, UK). There was no visual evidence of fungal infection on any of the treatments at the time of harvest.

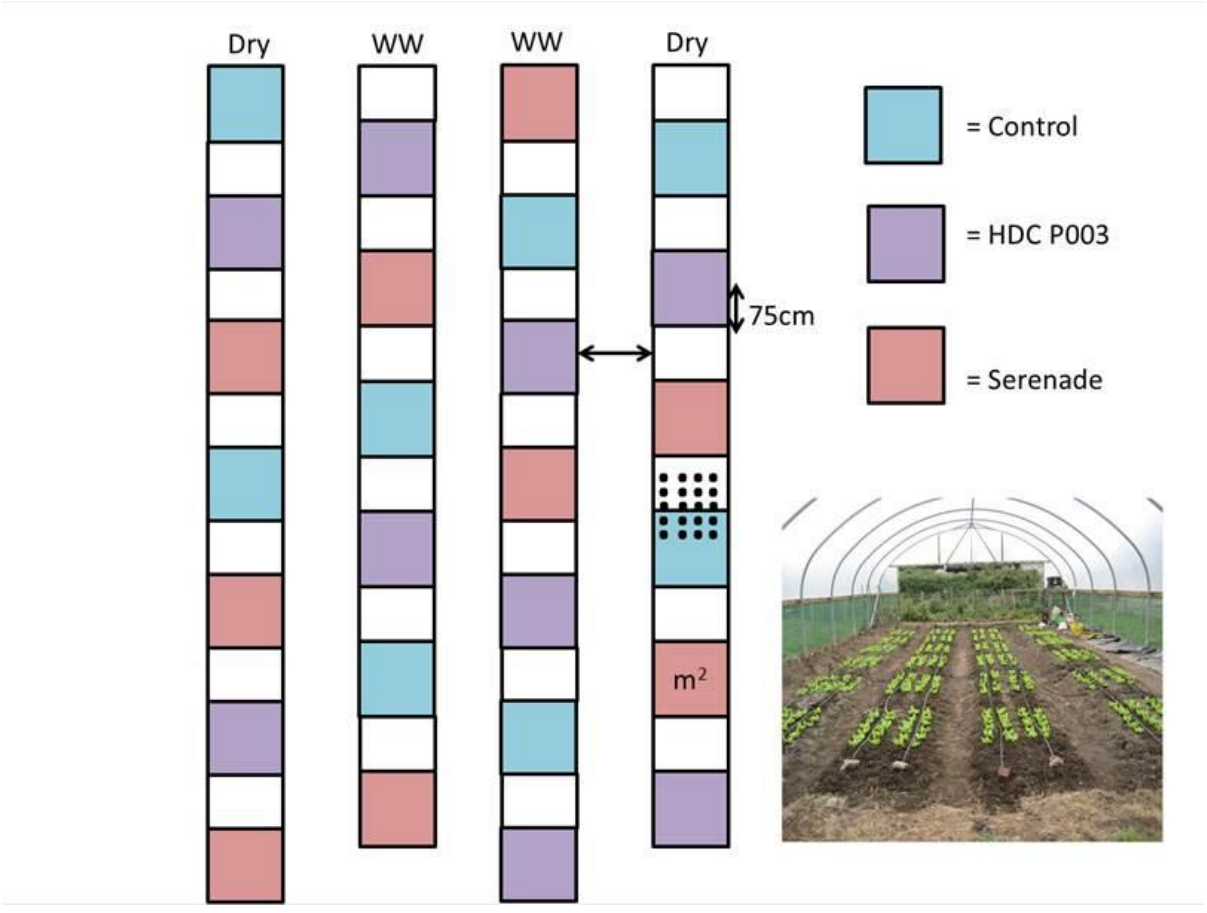


Figure 5: Field Layout of Experiment 4, showing the 4 beds in a polytunnel comprising dry and well watered (WW) treatments.

Sequencing the 16S gene of HDC P003

This work aimed to provide a sensitive technique to quantify bacterial colonization of the root system. Since bacterial 16S probes are considered the standard measurement methodology, the 16S gene of *HDC P003* was sequenced. DNA was extracted first by the GenElute Bacterial Genome DNA Kit (Sigma, USA) and then heat shock treatment, to reduce the number of stages involved thus decreasing the possibility of contamination. Bacterial colonies were placed in sterile water for 5 minutes at 95°C followed by at least 5 minutes in ice. A 1.5 kbps product was obtained via traditional PCR using the primers based on the 16S sequence of *V. paradoxus* S110 (Han *et al.,* 2011) in Table 1. The PCR mixture was composed of the following components: 12.5 µL DreamTag Green master mix kit (Thermofisher, UK), 1 µL of each 16.3 µM primer, 5.5 µL RNA-free water and 5 µL 1/100 template. The PCR program consisted of 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute and a final extension phase at 72°C for 10 minutes. The resulting PCR products were diluted to 20 ng μL^{-1} for purification and sequencing at Eurofins MWG Operon (Germany) with the addition of 10 pmol μL^{-1} of primers in Table 1. Based on the resulting partial sequences, primers were designed to complete the gene and to confirm the sequence (Table 1).

Name of primer	Sequence	Origin	Product (bp)	Use
First/16S/S110F	AGAGTTTGATCCTGGCTCAG	*Variovorax paradoxus* S110 16S gene	1523	Amplifying 16S DNA for sequencing
First/16S/S110R	AGGTGATCCAGCCGCACCTT		1523	
First/16S/S110R	AGGTGATCCAGCCGCACCTT		992	Sequencing 16S gene
First 20 bps R	ATTCCGATTAACGCTTGCAC		439	
16S/A8/Beg/F	CTGGCTCAGATTGAACGCTAG	Sequenced HDC P003 16S gene	974	Sequencing 16S gene
16S/A8/Mid/R	GGTGTTCCTCCGCATATCTAC		656	
16S/A8/End/R	CGGCTACCTTGTTACGACTTC		895	Sequencing 16S Gene

Table 1: Primers used in eludicating the 16S gene of *HDC P003*.

Detecting HDC P003 in root samples taken from field trials

Root samples of lettuces treated with *HDC P003* and broth or sterilized *HDC P003* were collected from Experiments 2 and 3 and DNA extracted using the Ultra Clean Soil DNA isolation kit (Mo-Bio, USA). Real time PCR was conducted following the protocol and 16S primer design in Bers *et al* (2011), using the Bio-Rad CFX96 machine (UK). Internal control used to account for the inhibitory factors of soil was pUC19 (Thermofisher, UK), with primers designed by Mäde *et al* (2008).

Sequencing the ACCd gene of HDC P003

Due to the difficulty in amplifying and sequencing *HDC P003* with primers based on *S110*, the more established ACCd gene in the *5C-2* strain was used as the basis for primer design (Table 2), which varies from the equivalent gene in S110 by an identity value (percentage of bases in the same alignment) of 91%. The ACCd gene of *HDC P003* was amplified by traditional PCR using the primers in Table 2 and the same conditions as for the 16S gene. The resulting 900 bp product was prepared for purification and sequencing at Eurofins MWG Operon (Germany) as before with the same primers used in PCR (Table 2).

Name of primer	Sequence	Origin	Product (bp)	Use
5C2/Ach/Accd/F	CAAGCGCGAGGACTGCAA	*Variovorax paradoxus 5C-2* ACCd gene	914	Amplifying ACCd DNA for sequencing
5C2/Ach/Accd/R	CCGTTGCGGAACAGGAAG		914	
5C2/Ach/Accd/F	CAAGCGCGAGGACTGCAA		861	Sequencing ACCd gene
5C2/Ach/Accd/R	CCGTTGCGGAACAGGAAG		856	
5C2/Ach/Accd/F	CAAGCGCGAGGACTGCAA		113	Detecting *HDC P003* ACCd gene in soil
2nd/5C2/ACCd/RRT	CGATCGACACCAGCGTGT		113	

Table 2: Primers based the ACCd gene.

Optimising the detection of *HDC P003* from root samples

The primers listed in Table 2 were originally tested with the same real time PCR protocol as with 16S, in the same machine, except that the anealing tempature was lowered to 53°C for 20 minutes. The PCR mixture for standards and negative controls consisted of: 5µL iTaq Universal SYBR Green Supermix (Bio-Rad, UK), 0.3 µL of each 10 µM primer (Eurofind WMG Operon, Germany), 2.9 µL DNase/RNase free water and 1.5 µL of template (diluted to 1/10 as with 16S). For the positive control, the mixture was the same, other than that the volume of DNase/RNase free water was adjusted to 2.4 µL, to account for 0.5 µL pUC19 (diluted 1/10). All standards, positive controls and negative controls were tested in triplicate. Due to the prevalence of primer dimers and non-specific amplification emitting fluorescence, a lower concentration of primer was tested (1 µM), which removed the former, but the melt peak (fluorescence) was too low. Increasing the annealing temperature and extending each stage of the protocol, so that it was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s, only increased the melt peak slightly. There were still primer dimers in the negative control and possible non-specific binding. The additon of 5% dimethyl sulfoxide (DMSO) (Simonović *et al.*, 2012) reduced, but did not completely eradicate, primer dimers in an inoculated Red Coral root sample. The melt peak is more pronounced (which means no non-specific binding) but still low. This was achieved by 0.5 µL neat DMSO to standards and positive control and decreasing water to 2.4 µL and 2.1 µL

respectfully. Gel were germinated over 3-4 days in a petri dish containing 20 mL of the treatment or a control (MSR and/or electrophoresis of the real time PCR products confirmed the correct region of 113 bp is amplified, with no amplification in the negative control.

Culturing HDC P003 for pot trials

This work aimed to determine an appropriate carrier medium for, and concentration of, *HDC003* for inoculation of lettuce seeds. Several pot trials were carried out using iceberg seeds that were germinated over 3-4 days in a petri dish comprising 20 mL of inoculant or water (see Table 3). In Trials 1 and 2, *HDC P003* was grown overnight in Modified Strullu Romand (MSR) broth at 28°C and 250 rpm on a shaking plate. The OD_{600} of the resulting bacterial culture was measured (which varied after each incubation) and the concentration diluted to an OD_{600} of 0.2 (10^9 CFU mL^{-1}) and 0.02 (10^8 CFU mL^{-1}) in MSR before addition to the petri dish. Iceberg seeds did not germinate in TSB, but bacteria grows to a higher concentration in this medium than in MSR, therefore, in Trial 3 *HDC P003* was grown in TSB and diluted to an OD_{600} of 0.2 (10^9 CFU mL^{-1}) in MSR.

Once germinated, 10 seedlings of each treatment were transplanted to a 50/50 (v/v) mixture of a non-sterile loam soil and sand (Alty's, UK) in 1 L pots (113 mm x 99 mm (top) / 81 mm (base)). No additional fertiliser was added to any treatment. Each treatment was divided in 2 trays (each comprising 5 pots) which were randomised in a naturally lit greenhouse (at Lancaster Environment Centre) and their location changed every 2-3 days throughout the experiment. In no treatment was there a significant tray effect, indicating that frequent re-randomisation of trays within the greenhouse ensured all plants were exposed to similar environmental conditions. Pots were weighed every 2-3 days to ensure that watering replaced evapotranspirational losses, with the same irrigation volume added to each pot in each trial (Table 3). Pot Trials 1, 2 and 3 were harvested after 6, 5 and 4 weeks respectively.

Experiment (Irrigation Volume in L)	Control	Control	*HDC P003* Inoculation (Low)	*HDC P003* Inoculation (High)
Trial 1 (1.82 L)	Water	MSR	OD_{600} 0.02 (10^8 CFU mL^{-1})	OD_{600} 0.2 (10^9 CFU mL^{-1})
Trial 2 (1.72 L)		MSR	OD_{600} 0.02 (10^8 CFU mL^{-1})	OD_{600} 0.2 (10^9 CFU mL^{-1})
Trial 3 (1.51 L)	Water	MSR		OD_{600} 0.2 (10^9 CFU mL^{-1})

Table 3: Treatments applied in pot trials.

Statistical Analysis

Statistical differences were calculated by T-tests in Excel (Microsoft) when comparing two factors and by an ANOVA in SPSS (IBM) for more than 2 variables.

Results

In Experiment 1, inoculating Iceberg lettuce with *HDC P003* (in broth) significantly ($P < 0.001$) increased yield by 19% (Table 4).

	Inoculated	Control	% change	P Value
Lettuce Head Weight (g)	890 ± 15	750 ± 16	+ 19 %	<0.001

Table 4: Head weight (g) of Iceberg lettuce. Data are means ± SE of 55 replicates

In Experiment 2, inoculating Endive with *HDC P003* (in broth) significantly ($P = 0.028$) decreased yield by 18% compared to the untreated control. In contrast, both Red Coral and Red Salanova produced consistently higher yields when inoculated with *HDC P003*, when compared to lettuces treated with broth alone (Fig. 6), with increases of 13% and 8% for Red Coral and Red Salanova respectively. These effects seem to depend on the presence of live *HDC P003*, since addition of broth (the carrier) had no significant effects on lettuce growth. No significant effects of Serenade were detected, irrespective of whether it was sterilized or not prior to application. Disease was largely absent from this experiment, thus disease incidence was not quantified.

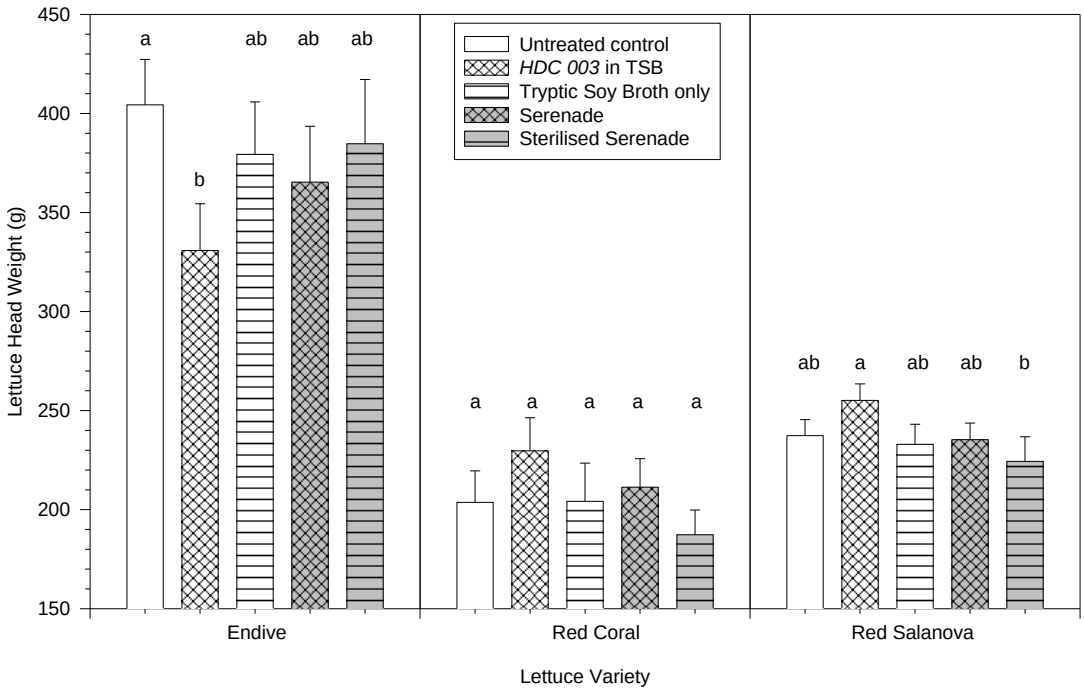


Figure 6: Head weight of lettuce inoculated with various treatments. Data are means + SE of 15 replicates, with different letters above the bars indicating significantly different (Tukey's HSD, $P < 0.05$) means within each panel.

In Experiment 3, there were significant treatment differences in both cultivars and with both inoculation techniques (Fig. 7). When treatments were applied to seedlings in propagation, sterilised Serenade increased head weight (by 7%) more than sterilised *HDC P003* in both Cook and Apollo. When *HDC P003* was applied directly to lettuce post-planting, head weight was increased 8% compared to uninoculated plants. Application of sterilised Serenade also increased lettuce head weight (by 5%). When data were combined across both cultivars and inoculation techniques, there were significant (P=0.019) differences between treatments, with the addition of sterilised Serenade producing the highest lettuce head weight overall.

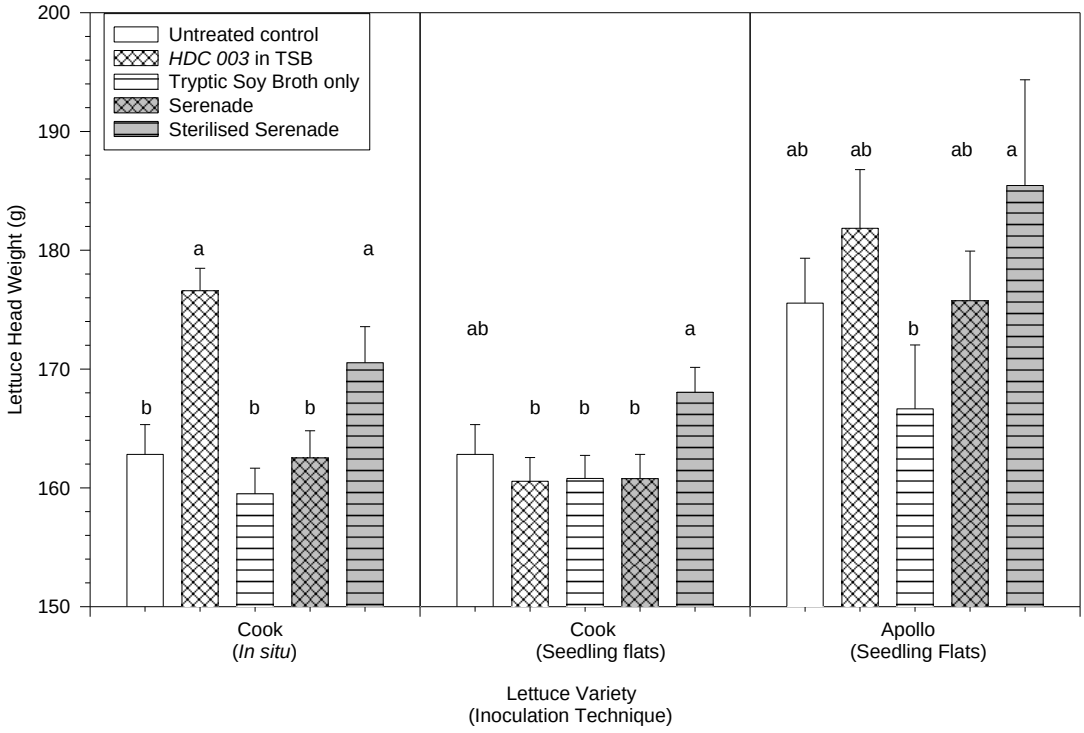


Figure 7: Head weight of lettuce inoculated with various treatments. Data are means + SE of 90 (Cook) and 20 (Apollo) replicates, with different letters above the bars indicating significantly different means within each panel (Tukey's HSD, $P < 0.05$).

In Experiment 4, automatic irrigation of the well watered treatments maintained soil water content (θ) close to field capacity throughout the experiment (data not shown). However, the non-irrigated treatments (similar to a commercial approach of irrigating to field capacity and avoiding unnecessary irrigation, particularly late in the season) had the highest fresh and dry weight (Table 5).

Treatment	θ at harvest (0-10 cm) vol / vol (%)	θ at harvest (15-25 cm) vol / vol (%)	Fresh Weight (g)	Dry weight (g)
Well Watered				
Control	31.1 ± 1.6^{a}	33.8 ± 1.2^{a}	210.3 ± 8.0^{c}	17.6 ± 0.8^{b}
HDC P003	33.3 ± 0.9^{a}	34.5 ± 0.7^{a}	209.3 ± 7.7^{c}	17.8 ± 0.6^{b}
Serenade	31.1 ± 1.0^{a}	33.8 ± 1.3^{a}	187.3 ± 8.4^{c}	16.0 ± 0.8^{b}
Dry				
Control	18.1 ± 1.5^{b}	21.2 ± 2.3^{b}	306.7 ± 8.2^{a}	23.3 ± 1.0^{a}
HDC P003	17.3 ± 1.8^{b}	20.6 ± 1.7^{b}	295.3 ± 7.7^{ab}	23.0 ± 1.2^{a}
Serenade	16.7 ± 2.3^{b}	19.2 ± 1.9^{b}	259.3 ± 11.4^{b}	21.7 ± 1.1^{a}

Table 4: Lettuce head weight and soil water content (θ) of the corresponding plots.

Data are means ± SE, with different letters indicating significant ($P < 0.05$) differences according to Tukeys test.

Quantifying HDC P003 on roots of inoculated lettuce

The resulting sequence varied from the 16S gene of *V. paradoxus* S110, as shown from the sequence alignment (Fig. 8), with an identity value of 86%. According to Basic Local Alignment Search Tool (BLAST), it shares the highest identity value with *Achromobacter xylosoxidans* and *Alicaligenes xylosoxidans*, so the exact genus of the bacterium cannot yet be determined.

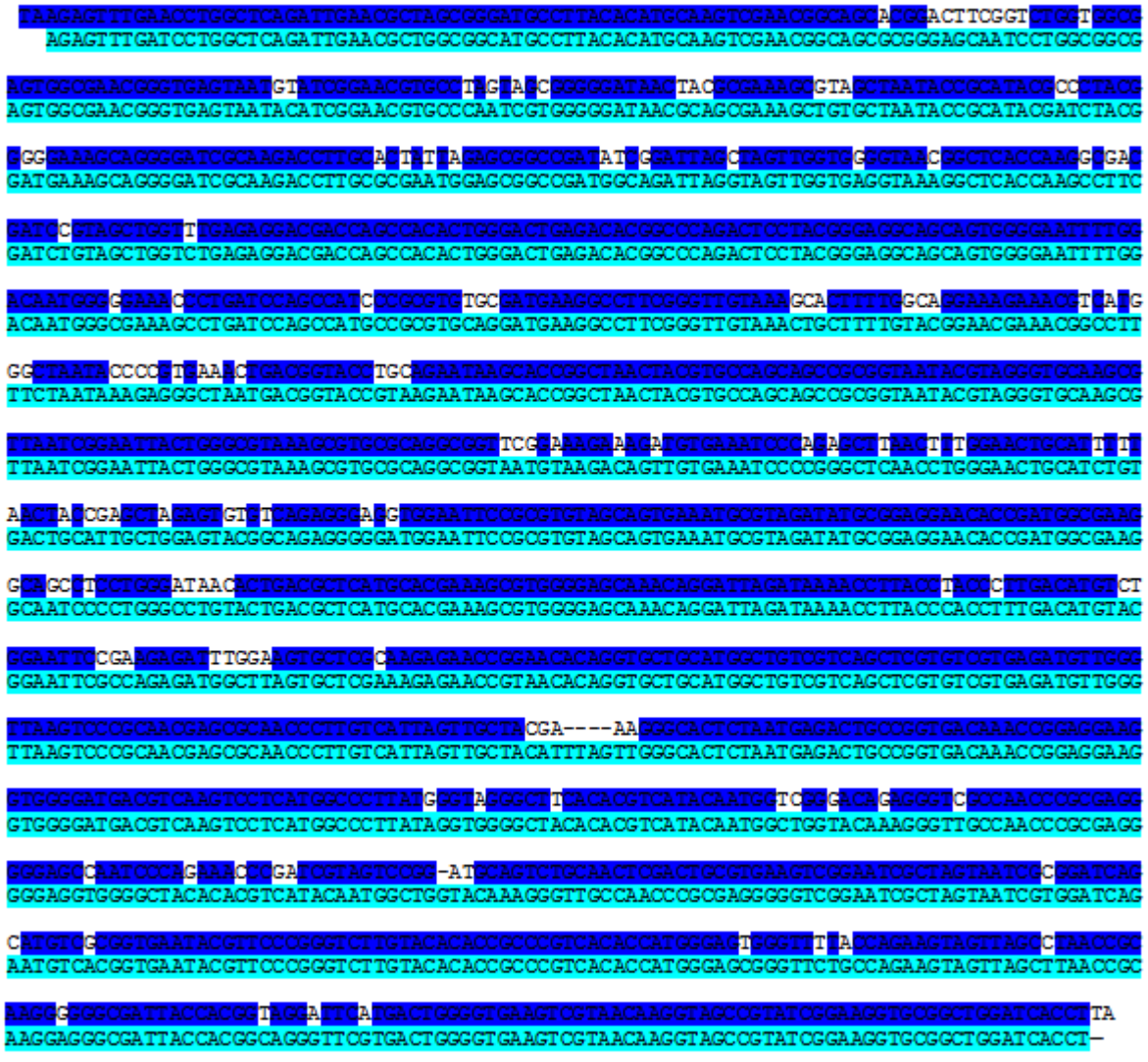


Figure 8: Alignment of the 16S genes of *HDC P003* against that of S110. *HDC P003* is the top sequence and dark blue where it is identical to 16S gene of S110 (light blue).

Roots treated with *HDC P003* and broth alone tested positive using primers described for real time PCR (Bers *et al* 2011). BLAST (NCBI) analysis of the oligos revealed these are not

specific to the *Variovorax* genus, indicating that further work developing more specific primers is necessary to allow detection of *HDC P003* on lettuce roots.

The ACCd sequence obtained (Fig. 9) so far from *HDC P003* has an identity value of 79% with *5C-2* and 80% with *S110*. The highest identity it shares is with *Achromobacter xylosoxidans* at 99%.

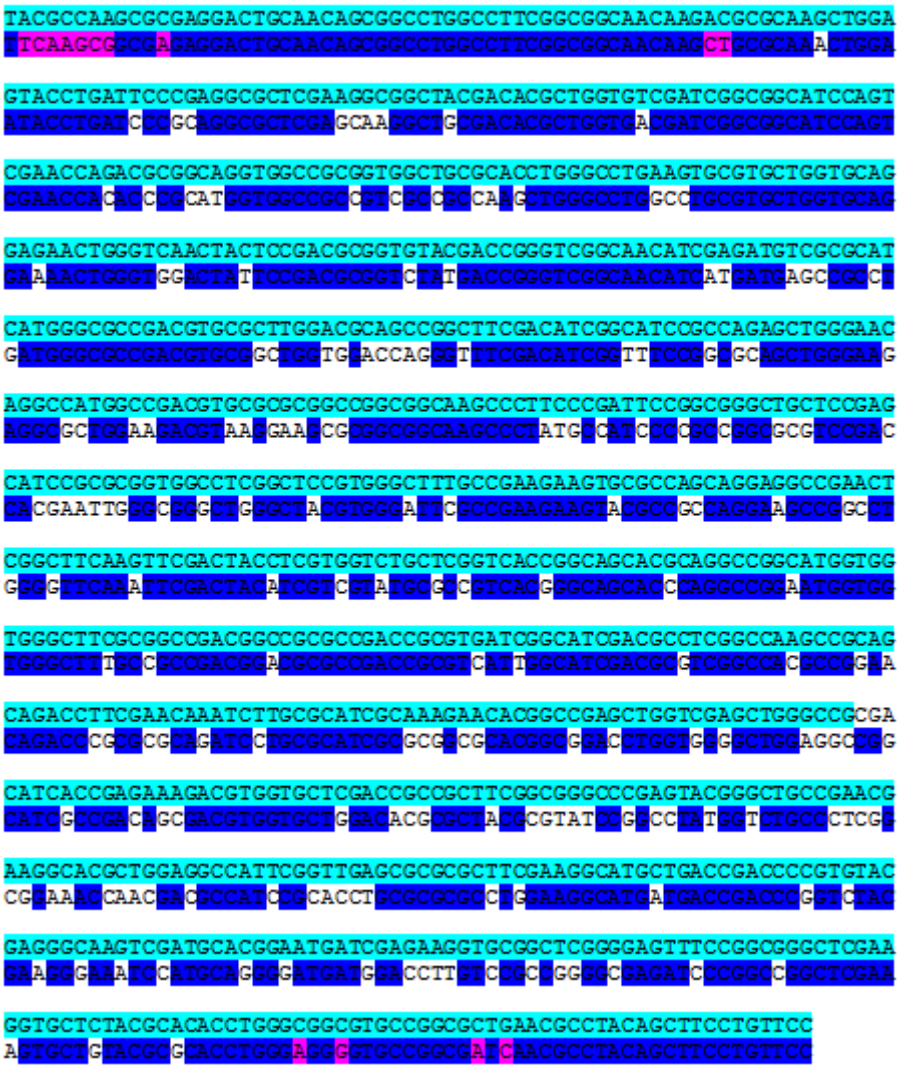


Figure 9: Alignment of the ACCd genes of *HDC P003* against that of 5C-2. *HDC P003* is the bottom sequence and dark blue where it is identical to 16S gene of S110 (light blue) and pink where more sequencing is required to ascertain the sequence.

Assessing HDC P003 in pot trials

In Trial 1, there were no significant treatment differences in lettuce head weight (data not shown). In Trial 2, plants treated with *HDC P003* at an OD_{600} of 0.2 (10^9 CFU mL^{-1}) had significantly ($P < 0.05$) greater weight and leaf area (22-23%) than plants treated with TSB alone (Fig. 10). Since all plants received the same irrigation volume (Table 3), water use efficiency was also higher.

In Trial 3, inoculation with *HDC P003* at an OD_{600} of 0.2 (10^9 CFU mL^{-1}) significantly increased leaf area when compared to either water or MSR controls (Fig. 11). Compared to both controls, inoculation increased leaf area, water use efficiency and shoot fresh weight by 20-27% (Fig. 12).

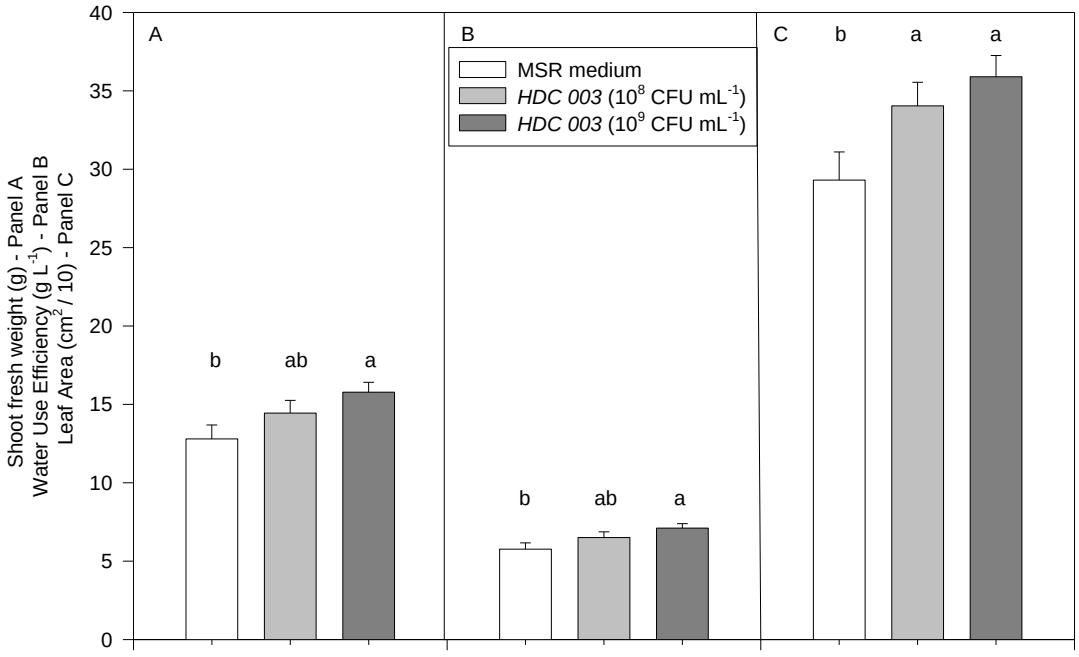


Figure 10: Fresh head weight (A), water use efficiency (B) and leaf area (C) of Iceberg lettuce in Trial 2. Data are means + SE of 9-10 replicates, with different letters above the bars indicating significantly different means within each panel (Tukey's HSD, $P < 0.05$).

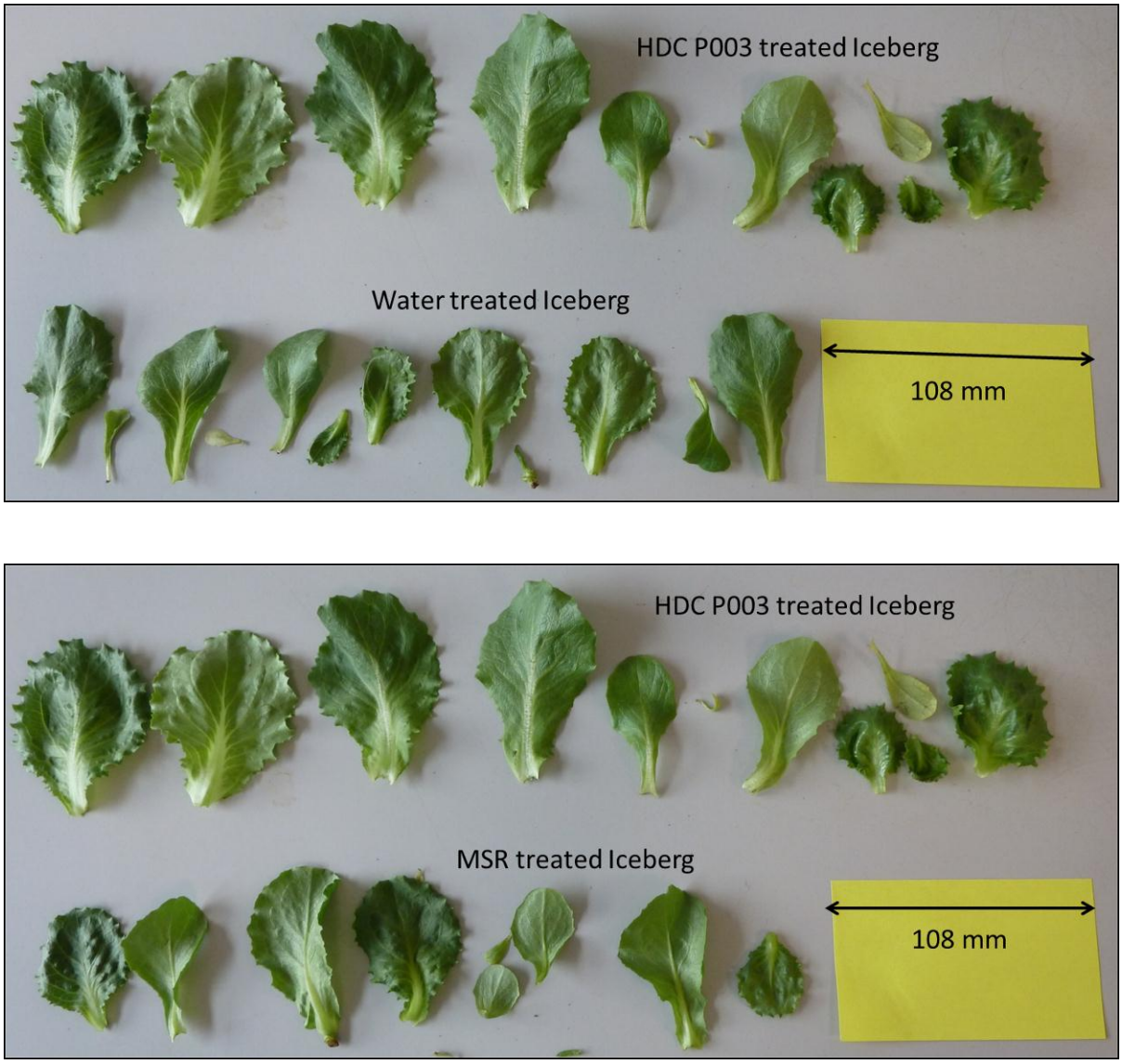


Figure 11: Individual leaf areas of representative plants from each treatment in Trial 3.

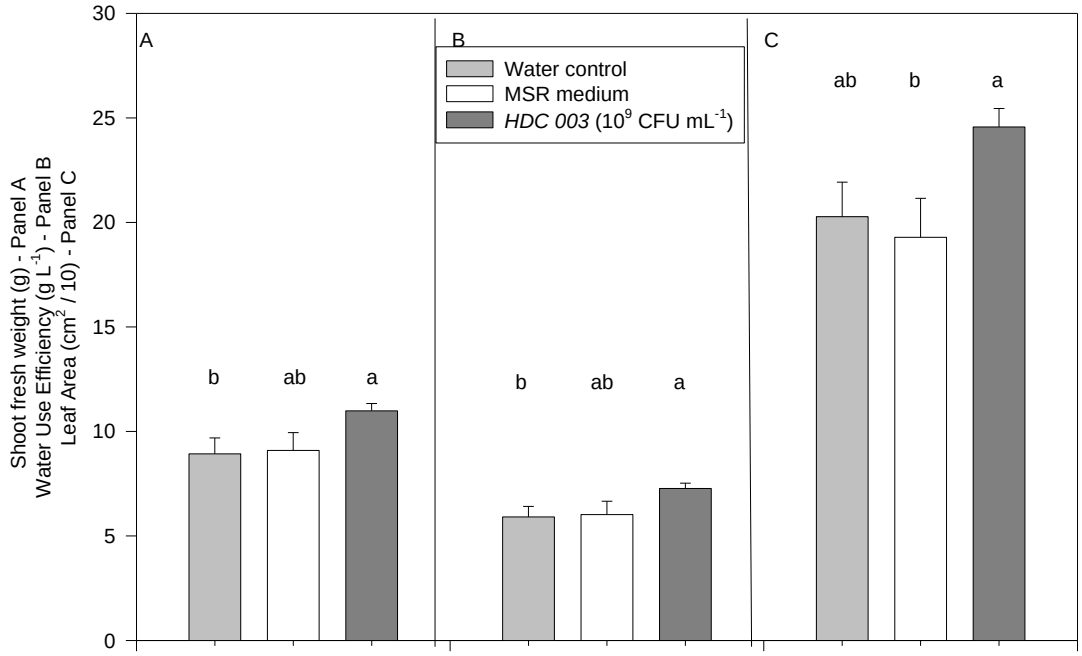


Figure 12: Fresh head weight (A), water use efficiency (B) and leaf area (C) of Iceberg lettuce in Trial 3. Data are means + SE of 8-9 replicates, with different letters above the bars indicating significantly different means within each panel (Tukey's HSD, $P < 0.05$).

Discussion

Physiological effects of *HDC P003* seemed to depend on both cultivar and application technique. In Experiment 2, direct inoculation of seedlings in the field decreased yield of Endive but consistently increased yield of both Red Coral and Red Salanova. Similarly, applying *HDC P003* directly to plants in the field increased lettuce head weight of Cook in Experiment 3. Although addition of rhizobacteria to propagation seedlings in the nursery may represent an easier method of application for the grower, results were less consistent. Only in Experiment 1 did the application of *HDC P003* significantly increase lettuce head weight (by 19%) when the treatment was applied in this way.

The addition of sterilised Serenade in Experiment 3 significantly increased head weight in both cultivars. Since autoclaving would have killed the live *Bacillus*, this may have freed nutrients present in the medium for plant growth. Where Serenade was applied according to the manufacturer's instructions (live *Bacillus)*, no effect on lettuce head weight was observed. The Serenade treatment should have also improved growth by reducing fungal infection, but positive effects did not occur, possibly since disease incidence was low.

Generally, data from the 3 field experiments show that *HDC P003* increases lettuce head weight. Since plants were grown under commercial conditions, “untreated” plants of cv. Cook in Experiment 3 actually had the microbial commercial stimulant Biagro (Glenside) added, which is a mixture of over 25 microorganisms, including nitrogen fixers *Rhizobia* and *Azotobacter*, mycorrhizal fungi which enhance plant phosphorous uptake and another species of *Bacillus*. When plants were treated with *HDC P003* (independent of the presence of Biagro) in the field, they grew larger than the “untreated” (commercial) control, possibly because the mechanism of growth promotion (ACCd decreasing stress-induced ethylene production) does not occur in the microbial cocktail Biagro. Although demonstrating compatibility between microbial products is essential prior to the addition of a new inoculant to the market, it suggests that addition of *HDC P003* (in isolation) is more effective at promoting growth than adding the complex microbial cocktail that is Biagro.

Pot Trials 2 and 3 showed that Iceberg lettuce yield increased proportionally to the concentration of *HDC P003* applied, with the optimal dosage being that added in Experiment 1 (10^9 CFU mL^{-1}). Crop water use efficiency improved with the highest concentration of *HDC P003*. Although this was a result of greater crop yield at the same irrigation volumes, it suggests that growers may be able to apply less water to achieve the same head weight of lettuces treated with *HDC P003.* Similar percentage increases in lettuce head weights occurred in both pot trials and the field, further suggesting that effects of *HDC P003* are cultivar dependent. Differences in cultivar response may be attributed to variation in the ability of *HDC P003* to colonise lettuce roots. A previous study found colonisation of lettuce roots by *Escherichia coli* varied according to the cultivar, with a difference of almost 700 relative light units for bioluminescence and 700 CFU mg^{-1} of root between the lowest and highest colonised cultivars, Valia and Dazzle (both Cos types) respectively (Quilliam *et al*., 2012). Consequently, this project aimed to develop molecular probes to investigate colonization of the root system by *HDC P003.*

Comparing the real time PCR primers (Bers *et al* 2011) to the *HDC P003* sequence does not reveal a complete match (Fig. 13), therefore the probe may not be amplifying the 16S gene of *HDC P003*. Additional work is being undertaken to sequence some of the ACCd gene of *HDC P003*, which appears to be more specific, with rather lower identity values than the 16S gene. Based on this, primers will be designed for real time PCR.

GCCTAGCAAGCGGGGATAACTACGCGAAAGGGTAGCTAATACCGCATACGCCCTACGGGGAAAGCAGGGGATCGCAAGACCTTGCGATTATTAGAGCGGCCGAT
GCCCAATCGTGGGGGATAACGCAGCGAAAGCTGTGCTAATACCGCATACGATCTACGGATGAAAGCAGGGGATCGCAAGACCTTGCGCGAATGGAGCGGCCGAT

Figure 13: Alignment of real time PCR primers (yellow) (Bers *et al* 2011), with the reverse oligo converted to its complementary sequence (on the right) against *HDC P003* (top and dark blue where aligned to S110). S110 is light blue.

The high identity values of the ACCd gene between the two strains of *Variovorax*, and *HDC P003*, points to the genes having the same function, which is likely responsible for stimulating growth of inoculated plants in field and pot trials. The rest of the ACCd gene will be sequenced using primers based on the portion of the gene already obtained. Combined with information gained from sequencing the 16S gene, this will enhance our understanding of the mechanisms by which *HDC P003* promotes lettuce growth.

The region where the forward primer aligns will have to be further sequenced, but the area homologous to the reserve primer is only one base out and therefore more specific than that of the tested 16S gene (Fig. 14). This is supported by bands of the correct size in gel electrophoresis produced by the real time PCR products, showing that the primers are amplifying the ACCd gene. Therefore primers 5C2/Ach/Accd/F and 2nd/5C2/ACCd/RRT in Table 2 can be used as probes to identify and numerate *HDC P003* on plant roots.

Figure 14: Real time PCR reserve primer aligned (red) with ACCd gene of *HDC P003* (dark blue).

Further optimising of the real time PCR protocol may be needed, such as trying lower concentrations of primers and increasing the annealing temperature to remove primer dimers. Soil contains inhibitors of PCR, which may have not been completely removed by DNA extraction, and therefore diluting the samples further to decrease the concentration of these compounds may improve output. These optimisation procedures will increase the chances of successfully detecting *HDC P003* on the roots of lettuce plants in the field, and all samples collected from field trials will be tested.

Although this research provides further evidence of the efficacy of a rhizobacterial mode-of-action in stimulating plant growth (by conducting experiments under commercial conditions), several barriers remain to the uptake of this inoculant by the industry. Firstly, the shelf-life of the bacteria in different media (TSB, MSR) needs to be measured by storage at room temperature (like other commercial products such as Serenade). Secondly, procedures are

necessary to mass produce the organism (e.g. by bio-fermentation), in such a way that the correct concentration is always attained and kept for a long period during storage. Lastly, commercial licensing of this inoculant as a growth stimulant requires considerable financial investment.

Conclusions

Although the effects of *HDC P003* on lettuce depended on cultivar and inoculation technique, growth stimulation was usually demonstrated. Direct comparison of mode of application in Experiment 3 demonstrated that rhizobacterial application post-planting was more effective at promoting growth. Further trials should seek to identify the most appropriate (carrier) medium for bacterial proliferation.

Technology Transfer

Results from this project were disseminated at the Association of Applied Biologists Positive Plant-Microbe Interactions conference (Lincolnshire, 2-3 December 2013)

IC Dodd, AA Belimov, ED Elphinstone, L Chen, RG Teijeiro, C Kemp, H Fielding, H Wright (2013) Exploiting rhizobacteria that mediate plant hormone status. *Aspects of Applied Biology* 120, 29-34. (Accompanied by oral presentation)

CL Kemp, IC Dodd (2013) Inoculation with the ACC-deaminase containing rhizobacterium *Variovorax paradoxus* 3C-1 increased lettuce yield under UK field conditions. *Aspects of Applied Biology* 120, 101-105. (Accompanied by poster presentation)

Additionally, an article was prepared for HDC News to announce the project (published in February 2014 issue), and a further one is in preparation (planned for April 2014 issue).

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