Project title	The use of Rhizobacteria to enhance the shelf life of pot plants
Project number:	PC 282
Project leader:	Professor W J Davies
Report:	Final Report (March 2009)
Previous report	N/A
Key staff:	Ms Lin Chen Dr Ian Dodd
Location of project:	Lancaster University
Project coordinator:	Ms Cheryl Brewster
Date project commenced:	April 2008
Date project completed (or expected completion date):	March 2009

Key words: Pot and Bedding plants, shelf life, ethylene , rhizobacteria

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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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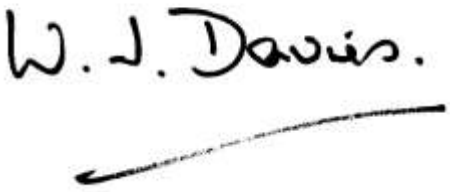

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Grower Summary

Headline

When added to substrates routinely used for the production of ornamentals, naturally-occurring soil bacteria increased the quality (and shelflife of a range of crops.

Background and expected deliverables

It is well accepted within the industry that increased ethylene levels in many crops arising from a variety of even mild stresses (e.g. substrate drying or waterlogging, substrate compaction and/or sleeving of crops including Poinsettia, New Guinea Impatiens and many bedding plants) results in leaf and/or flower drop and premature senescence of the crop.

R*hizobacteria* are a group of bacteria that exist in the rhizosphere, a zone that extends a few millimetres around the root hairs, which feed off root exudates. They are currently subject of considerable research as some strains exhibit a degree of bio-suppression of root diseases.

Researchers at Lancaster University and in St Petersburg in Russia have isolated particular strains of *Rhizobacteria* that enhance the performance of a range of crops in a variety of cultural conditions. The work has demonstrated that the enhanced shoot and root growth and crop health under drought conditions is linked to the disruption by the Rhizobacterium of ethylene production (stimulated in plants as a response to the stress).

Pot and bedding plants such as Poinsettia, geraniums and impatiens show enhanced ethylene generation under stress and subsequently high sensitivity to this ethylene, leading to the subsequent leaf/flower drop in transit and 'on-shelf' whilst sleeved - a major cause of wastage. By maintaining a healthy population of these *Rhizobacteria* strains in the substrates, it is proposed that the levels of ethylene can be reduced, minimizing ethylene- induced damage. The reduction of ethylene accumulation in the roots/substrate also improve the root growth in many plants, leading to improved plant vigour. Rhizobacteria may require certain substrates to produce sufficiently high population densities to alter ethylene production levels.

This project was designed to address the following issues of concern to the industry:

a) Establish conditions for pot plant production which are conducive to ethylene production.

b) Develop a method for the monitoring of establishment of the bacterial colony in the substrate.

c) Develop specific media necessary for the successful colonisation of the *Rhizobacteria*

d) Investigate the capacity of *Rhizobacteria* applied to media to restrict the synthesis of ethylene by pot plants, and the impact of time of application and impact of the dose applied,

e) Investigate the use of the bacteria with different plant cultivars.

Summary of the project and main conclusions

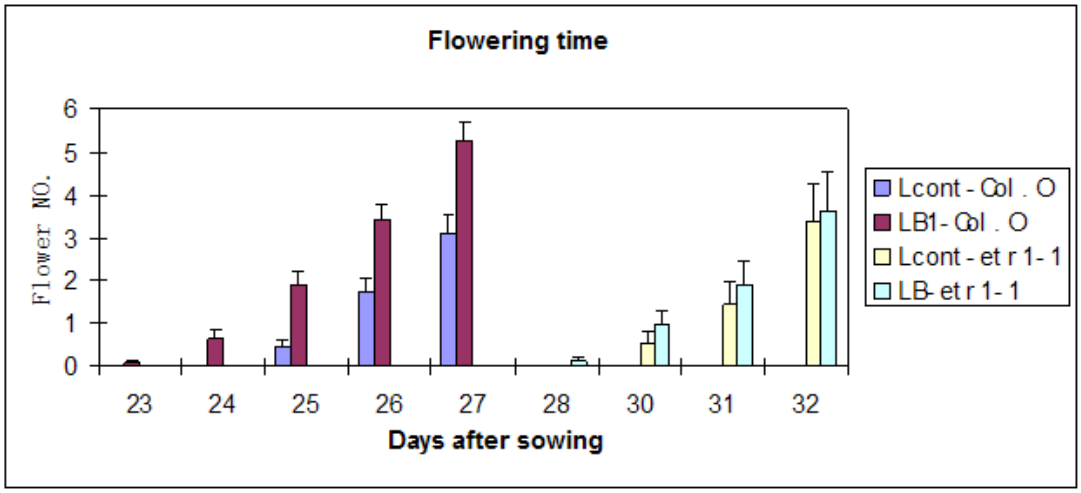
These experiments suggest strongly that rhizobacteria will colonise a range of substrates and can have potentially beneficial effects on quality of ornamentals. Our evidence suggests that these effects are related to a reduction in the accumulation of potentially damaging levels of the plant growth regulator ethylene. This compound is known to accumulate in response to a range of environmental stresses, particularly ozone, water deficit and waterlogging.

There must be excellent opportunities for the exploitation of these results in a commercial context. Bacteria might be used to allow reduced water use in plant production and to generally enhance the robustness of the production process against a challenging and changing climate.

An observation made while this work was in progress was that rhizobacteria promoted vegetative development of ornamentals such that plants flowered more quickly. In addition, this treatment increased numbers of flowers initiated. While this observation is not relevant to the deliverables of this project, clearly it is relevant to the industry. We are following up this work to try to understand the mechanistic basis of the work.

Experiments with the model plant Arabidopsis clearly show the response. Interestingly, there is no promotion of flowering in an ethylene sensitive mutant of Arabidopsis, suggesting that the response is ethylene based.

We recommend further investigation of this response which could be important for crop scheduling and for increasing flower load.



Financial benefits

Bacteria are low cost soil additives which would not add substantially to the cost of the plant production process but might increase plant quality, reduce wastage and enhance productivity.

Action points for growers

None at present. Negotiations are underway for the commercial production of rhizobacterial strains so that commercial scale trials may be undertaken.

Science section

Introduction

It is well accepted within the industry that increased ethylene levels in many crops arising from a variety of even mild stresses (e.g. substrate drying or waterlogging, substrate compaction and/or sleeving of crops including Poinsettia, NG Imps and many bedding plants) results in leaf and/or flower drop and premature senescence of the crop. R*hizobacteria* are a group of bacteria that exist in the rhizosphere, a zone that extends a few millimetres around the root hairs, which feed off root exudates. They are currently subject of considerable research as some strains exhibit a degree of bio-suppression of root diseases. Researchers at both the Lancaster Environment Centre (LEC) in Lancaster University and in St Petersburg in Russia have isolated particular strains of *Rhizobacteria* that enhance the performance of a range of crops in a variety of cultural conditions (Belimov et al. 2009). The work has demonstrated that the enhanced shoot and root growth and crop health under drought conditions is linked to the disruption by the Rhizobacterium of ethylene production (stimulated in plants as a response to the stress). These strains of *Rhizobacteria* have the ability to absorb and metabolise the precursor to ethylene, ACC, to form ammonia and ketobutyrate The constant absorption of ACC by the bacteria results in roots exuding increasing quantities of ACC into the root zone to maintain the equilibrium between external and internal levels, reducing overall ethylene production by the plant. Pot and bedding plants such as Poinsettia, geraniums and impatiens show enhanced ethylene generation under stress and subsequently high sensitivity to this ethylene, leading to the subsequent leaf/flower drop in transit and 'on-shelf' whilst sleeved - a major cause of wastage. By maintaining a healthy population of these *Rhizobacteria* strains in the substrates, it is proposed that the levels of ethylene can be reduced, minimizing ethylene- induced damage. The reduction of ethylene accumulation in the roots/substrate also improve the root growth in many plants, leading to improved plant vigour Rhizobacteria may require certain substrates to produce sufficiently high population densities to alter ethylene production levels.

This project was designed to address the following issues of concern to the industry:

a) Establish conditions for pot plant production which are conducive to ethylene production.

b) Develop a method for the monitoring of establishment of the bacterial colony in the substrate.

c) Develop specific media necessary for the successful colonisation of the *Rhizobacteria*

d) Investigate the capacity of *Rhizobacteria* applied to media to restrict the synthesis of ethylene by pot plants, and the impact of time of application and impact of the dose applied,

e) Investigate the use of the bacteria with different plant cultivars.

Materials and Methods

Objective a) Establish conditions for pot plant production which are conducive to ethylene production.

A range of experiments was conducted with plant material provided by Porters of Formby or by Garden Centre Plants of Preston. Plants were grown on under controlled environment conditions in the Lancaster Environment Centre and subjected to a range of environmental stresses expected to result in ethylene accumulation. Individual stresses are detailed in the sections below. Adverse effects of environmental stress on plant quality were always apparent but ethylene accumulation was not always detected.

The stress treatments

1) Ethylene. All the experiments where ethylene was applied were carried out in a closed system with a light transparent lid. Ethylene concentration in the closed space was 11.4ppm. Plants were kept in these systems for 24 hours.

2) Chilling. Antirrhinum plants were treated with cold for 3 days. During this time, the temperature was held at 20 C in the light and 10C in the dark. Plants were treated with cold 1month after bacterial inoculation.

3) Ozone. Antirrhinums were placed in ozone chambers or in chambers with clean air. The ozone levels were maintained around 60ppb (average concentration on warm UK summer day) for a week. Ethylene concentrations in the flowers were then determined.

4) Mechanical stress. Roots of Poinsettia plants were inoculated with bacteria. After 2 weeks, plants were sleeved for 2 days. Ethylene accumulation was then measured and subsequently, plant quality assessed.

5) Drought. Pots were fully watered and allowed to drain freely. The weights of the pots were recorded. The pot weights were recorded every day. For the control plants, the amount of water lost by the plants by transpiration was added to the soil, but only 50% of water lost was given to the drought-treated plants.

Objective b) Develop a method for the monitoring of establishment of the bacterial colony in the substrate.

This objective proved relatively easy to fulfill. The bacterium of choice for this project was *Variovorax paradoxus*, selected from a range of soils and known to be highly effective in breaking down ACC (the immediate precursor of ethylene (see above). Variovorax proves to be relatively resistant to antibiotics and so treating roots with antibiotic solutions was shown to reliably remove all other bacteria from roots, allowing this bacterium to be readily quantified. The efficacy of this simple treatment was tested by examining bacteria using microscopical techniques and using molecular typing (fingerprinting) to confirm the existence of pure cultures (data not shown). Details of this procedure are provided under methods for Objective c.

Objective c) Develop specific media necessary for the successful colonisation by Rhizobacteria

Uniform seedlings of a variety of spp. were transplanted into plastic pots (2 L, containing different growing substrates at field capacity: loam-based compost (John Innes No. 2, J. Arthur Bowers, Lincoln, UK), a peat-based compost (Levington's M3, Levington, UK), a

field soil from Lee Farm, Myerscough College, Bilsborrow, Lancs, and a wood-and peat-based substrate sourced from Bulrush Peat, Belfast.

Prior to transplanting, a bacterial suspension of *Variovorax paradoxus* (Strain 5C2) was added to the pots to a final concentration of 10^6 cells g^{-1} soil/substrate. (Bacteria were grown on agar BPF medium for 3 days at 28°C, cells collected from agar surface, to minimise transfer of nutrient-rich agar to the pots, and suspended in tap water with a final concentration of 10^8 cells ml^{-1}). Pots were maintained in a naturally lit greenhouse and watered daily. After four weeks, the plants were removed from the soil/substrate, roots were thoroughly shaken to remove adhering soil particles and homogenized in sterile tap water with sterile mortar and pestle. Homogenates were serially diluted in 10-fold steps and 50 µL aliquots were plated in two replicates on BPF agar supplemented with 30 µg ml^{-1} kanamycin and 20 µg ml^{-1} rifampicin (as explained above, *V. paradoxus* 5C-2 shows resistance to antibiotic). The BPF medium was additionally supplemented with 40 µg ml^{-1} nystatin to prevent fungal growth. The characteristic colonies of *V paradoxus* 5C-2, which were absent on plates containing homogenates of uninoculated roots, were counted after incubation at 30°C for 4 days. The data were expressed as the number of colony forming units (CFU) per gram of root fresh weight (FW).

To compare the effectiveness of soil inoculation (as described above) and seed inoculation in establishing bacteria on the roots, seeds were incubated in a well-aerated bacterial suspension (1–2 x 10^{10} CFU ml^{-1}) for 0, 3, 6 and 18 hours prior to transplanting.

Objective d) Investigate the capacity of Rhizobacteria applied to media to restrict the synthesis of ethylene by pot plants, and the impact of
time of application and impact of the dose applied.

In these experiments, ethylene was determined by gas chromatography. Excised tissue was immediately enclosed in glass test tubes and capped with a rubber serum stopper. Then the tubes were held at 23C for varying lengths of time. One-ml gas samples were periodically taken and analyzed for ethylene. The tubes were flushed with air outside the lab after each sample interval.

Objective e) Investigate the use of the bacteria with different plant cultivars

For experiments reported under Objectives d and e, the following cultural procedures were used:

Bacteria strains were cultured on solid Bacto-Pseudomonas F (BPF) medium at 28℃ for three days The composition of BPF medium included (g l^{-1}) peptone 10, casein hydrolysate 10, K_2HPO_4 1.5, $MgSO_4$ 1.5, agar 15. The bacterial solution for plant inoculation was prepared by scraping the bacteria from the agar plates into tap water. The liquid suspension culture was diluted with water to yield 10^7 cells ml^{-1}as determined by monitoring the optical density (OD) at 540nm (Ultrospec 2100 *pro* spectrophotometer Amersham company, UK.

The bacterial suspension was added to the surface of the pots with plants present. The final concentration of bacteria in the soil was 10^6 cells g^{-1}soil.

Results

Substrates and colonisation

Plants rooted into different substrates were irrigated with a standard bacterial suspension of *V. paradoxus* 5C2. Plants grown in John Innes No. 2 had the highest root colonization by *V. paradoxus* 5C2, soil from our field site the least and bacteria were absent from roots of plants grown in the wood-based substrate (Fig. 1). *In vitro* fungal growth was most prominent on plates with root homogenates from the latter substrate. Soil inoculation with a more concentrated bacterial suspension (X3) was necessary to detect any bacterial colonisation of roots grown in the wood-based substrate. The more concentrated bacterial suspension significantly increased root colonisation of plants grown in John Innes No. 2 and the field soil.

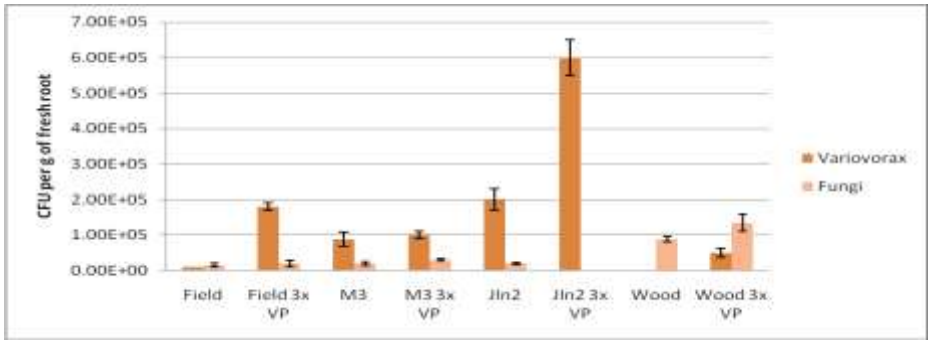


Fig. 1: V. paradoxus and fungal root colonization after plants were transplanted into different substrates that had been irrigated with a bacterial suspension.

Seed treatment also resulted in higher colonisation in John Innes No. 2 Soil from our field site showed the least colonisation and bacteria were absent from roots of plants grown in the wood-based substrate (Fig. 2).

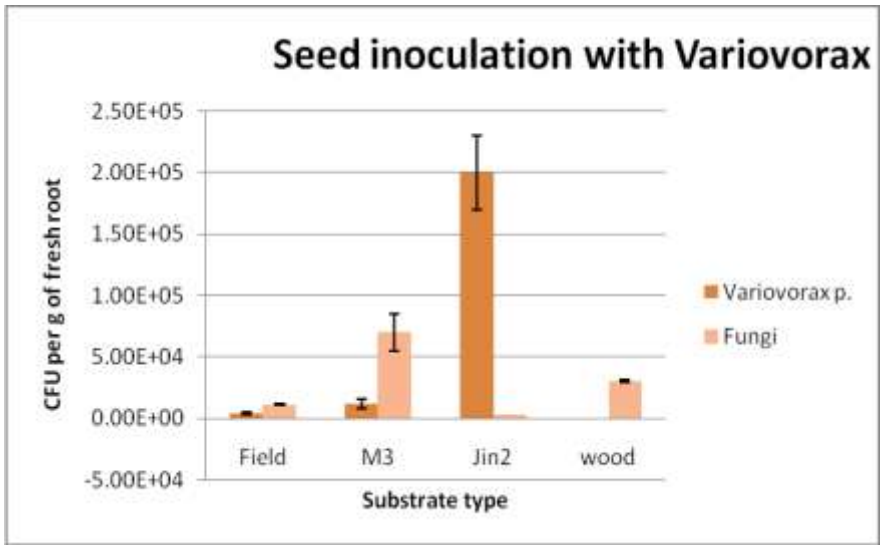


Fig. 2: V. paradoxus and fungal root colonization in different substrates after inoculation with a bacterial suspension.

As the duration for which seeds were incubated in bacterial suspension increased, bacterial root colonization increased (Fig. 3), but the percentage of seeds germinating decreased. Generally, irrigating the substrate with a bacterial suspension resulted in greater root colonization than incubating the seed with a bacterial suspension.

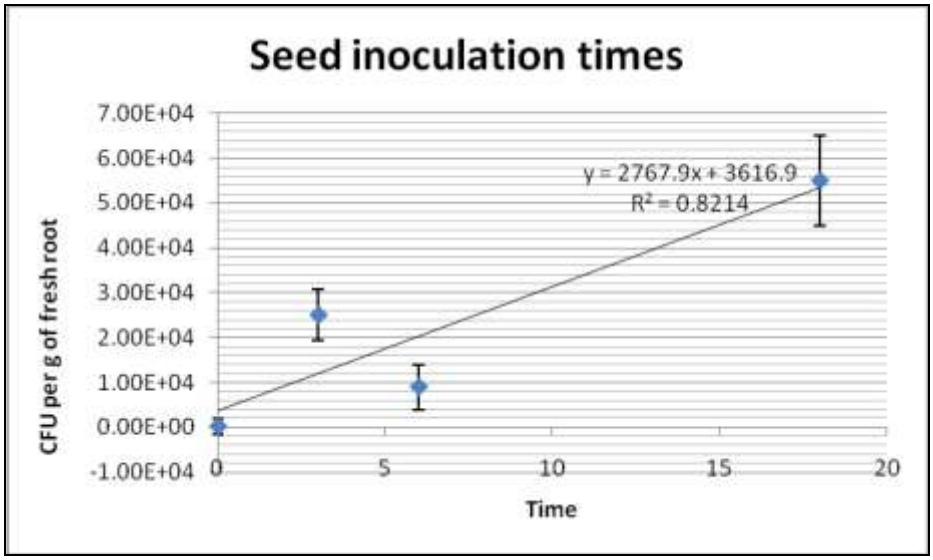


Fig. 3: Effects of duration of seed incubation on V. paradoxus root colonization when seeds were planted into a peat-based substrate (Levington's M3).

b) Stress treatments and plant responses

Treatment of Antirrhinums with 11.4 ppm ethylene results in abscission of around 60% of flowers and wilting of a proportion of those that do not abscise (Fig. 4). Interestingly, not all stresses increased ethylene levels in this plant. One stress that was particularly damaging was ozone and ethylene accumulation was promoted by this stress. Bacterial treatment was able to counteract this stress (Fig. 5)

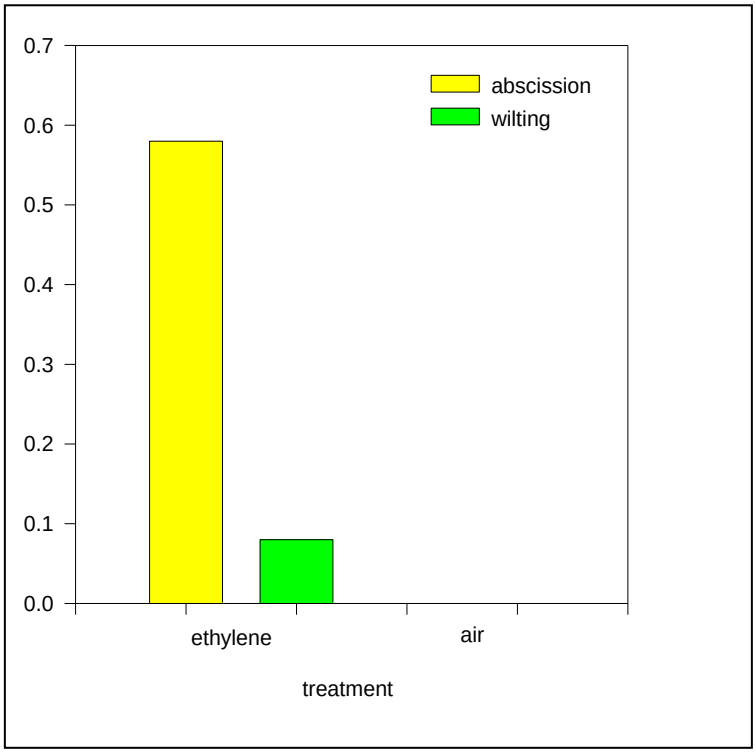


Fig. 4 Impact of ethylene treatment on flower quality of Antirrhinum

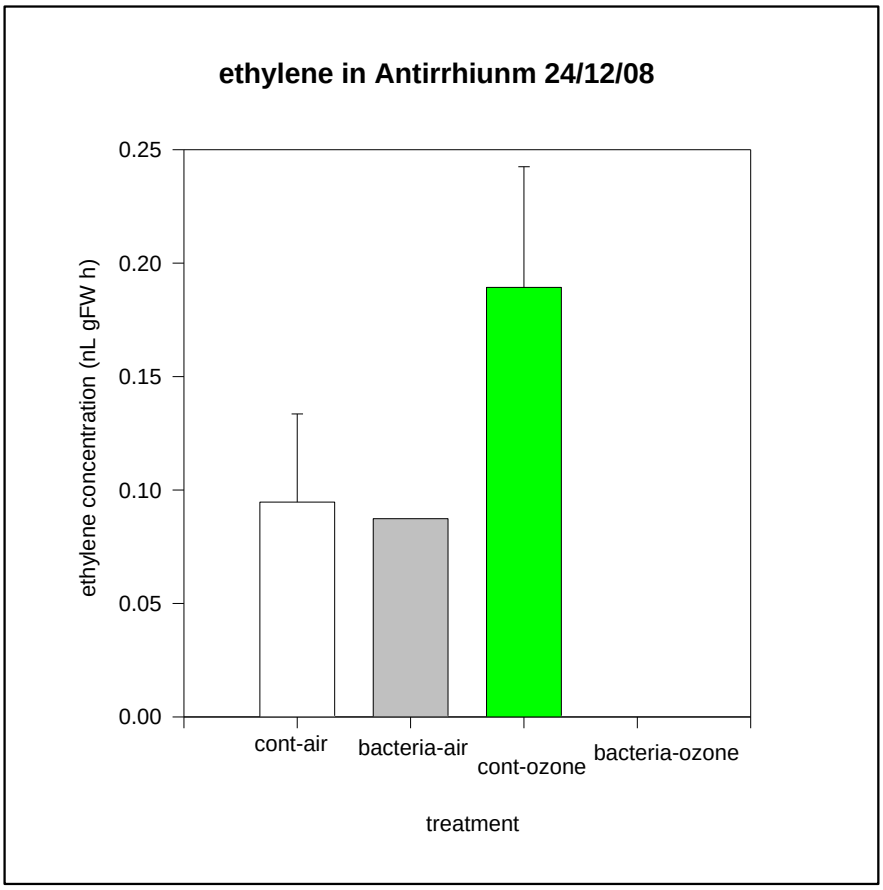


Fig 5. Ethylene accumulation as a result of treatment with 60 ppb ozone and the amelioration of this effect by treatment with Variovorax

These results are of interest in relation to observations on the effects of drought and other stresses on NG Imps (see below). Effects of low concentrations of ethylene have been observed in wide range of spp (see e.g. Fig. 6).

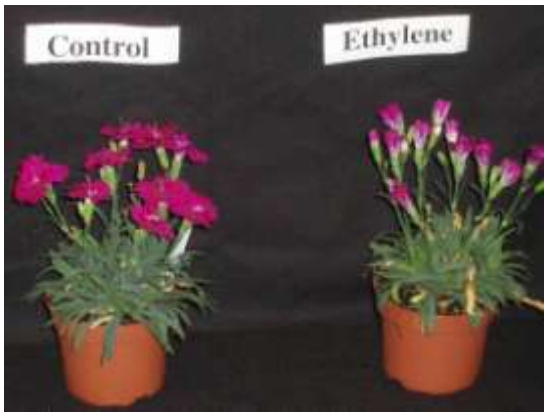


Fig.6. Effects of 11.4 ppm ethylene on flower quality of Dianthus

One of our most spectacular results was the clear amelioration by rhizobacterial treatment of drought effects on NG Imps (Fig.7). The mechanism behind this effect is discussed in the section below.

Fig. 7. Effect of drought on quality of NG Imps, ameliorated by bacterial treatment (plants on LHS of the figure)

Most of our treatments applied to Poinsettia had no impact on ethylene accumulation although we were able to detect low accumulations of ethylene, particularly in bracts. These accumulations could be reduced by bacterial treatment (Fig. 8), suggesting that there may be some scope for the use of bacteria to ensure high quality plant production.

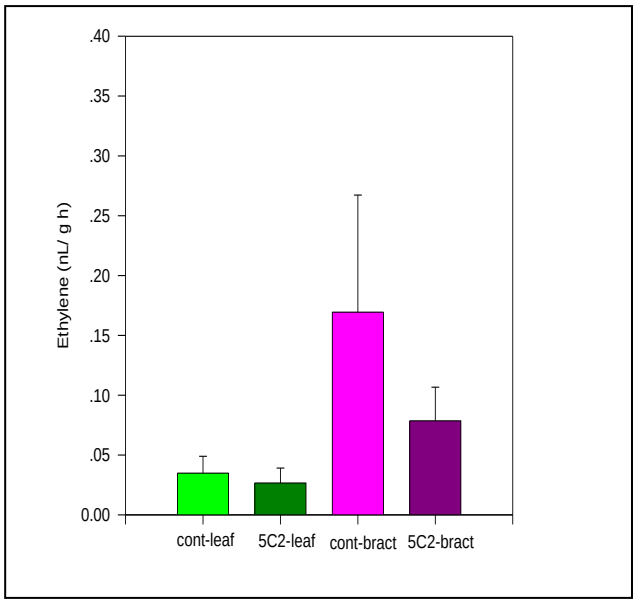


Fig. 8. Amelioration of ethylene accumulation in Poinsettia by rhizobacterial treatment

In several experiments there was a clear effect of rhizobacterial treatment on plant quality (Fig. 9).

Fig. 9. Effect of bacterial treatment on quality of stressed Poinsettia

Discussion

These experiments suggest strongly that rhizobacteria will colonise a range of substrates and can have potentially beneficial effects on quality of ornamentals. Our evidence suggests that these effects are related to a reduction in the accumulation of potentially damaging levels of the plant growth regulator ethylene. This compound is known to accumulate in response to a range of environmental stresses, particularly ozone, water deficit and waterlogging.

Our recent research has shown that

(1) increasing background O_3 has a much more pronounced effect on root than shoot biomass (Mills *et al*., in prep), with a potentially negative effect on plant robustness;

(2) O_3 can increase transpiration and reduce drought tolerance by altering hormonal (ethylene and ABA) regulation of stomata and leaf growth as controlled by both plants and plant-associated bacteria (Mills *et al.,* 2009; Wilkinson and Davies, 2009, unpublished data from LU); and

(3) there is a temporal biphasic response to O_3: initially (under hormonal control) a combination of more open stomata, greater initial leaf area (Mills *et al*., unpublished) and reduced root biomass will all contribute to a massive reduction in the control of plant water balance, eventually leading to secondary hydraulic limitations to growth giving rise to reductions in plant quality.

Impacts of other stresses may also be manifested via ethylene accumulation and ameliorated by soil additives.

Conclusions

There must be excellent opportunities for the exploitation of these results in a commercial context. Bacteria are low cost soil additives which would not add substantially to the cost of the plant production process but might increase plant quality, reduce wastage and enhance productivity. Bacteria might be used to allow reduced water use in plant production and/or to generally enhance the robustness of the production process against a challenging and changing climate. Bacteria may be particularly useful with plants growing in small volumes of substrate.

Technology transfer

We have opened negotiations with two chemical companies for the commercial production of rhizobacterial strains. We are now in a position to conduct commercial scale trials

References

Belimov AA, Dodd IC, Hontzeas N, Theobald JC, Safronova VI, Davies WJ (2009) Rhizosphere bacteria containing ACC deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling. *New Phytologist* 181, 413-423.

Mills, G., Hayes, F., Wilkinson, S. and Davies, W.J. (2009) Chronic exposure to increasing background ozone impairs stomatal functioning in grassland species. *Global Change Biology* (in the press)

Wilkinson, S, Davies WJ (2009) Ozone suppresses drought- and ABA-induced stomatal closure via an ethylene-dependent mechanism. *Plant Cell and Environment* (in the press).

Appendix

An observation made while this work was in progress was that rhizobacteria promoted vegetative development of ornamentals such that plants flowered more quickly. In addition, this treatment increased numbers of flowers initiated. While this observation is not relevant to the deliverables of this project, clearly it is relevant to the industry. We are following up this work to try to understand the mechanistic basis of the work.

Experiments with the model plant Arabidopsis clearly show the response (Fig. 10). Interestingly, there is no promotion of flowering in an ethylene sensitive mutant of Arabidopsis, suggesting that the response is ethylene based.

We recommend further investigation of this response which could be important for crop scheduling and for increasing flower load.

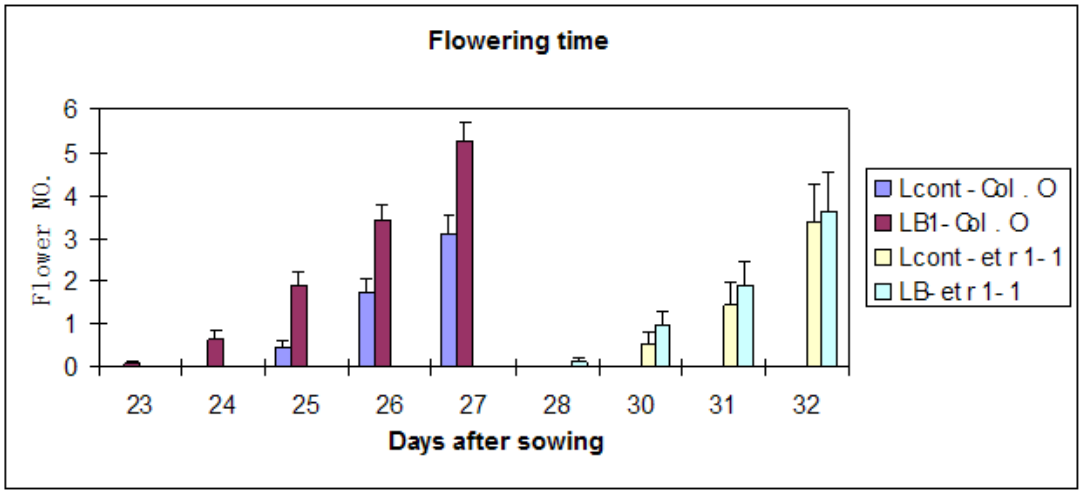


Fig. 10. Influence of rhizobacteria on flowering time and flower number of Arabidopsis wildtype (red and blue bars) and an ethylene insensitive mutant (yellow and cyan bars). Bacterial treatment (red bars) both speeds up and enhances flowering of wild type but (cyan bars) have no effect on flowering of the ethylene insensitive genotype