Appendix 3:

Physiological Effects of ACC deaminase Producing Rhizobacteria on *Brassica oleracea*

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Physiological Effects of ACC deaminase Producing Rhizobacteria on *Brassica oleracea*

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Abstract

Plant growth promoting (PGPR) rhizobacteria may ameliorate plant drought stress responses by altering plant hormone homeostasis, particularly those that interfere in plant ethylene relations (a hormone which inhibits plant growth in response to drought stress). Rhizobacteria which produce ACC deaminase (ACCd) inhibited ethylene biosynthesis *in planta* but there has been some conflicting data on the effects of ACCd producing PGPR on signalling of another phytohormone (ABA) in different species. Therefore, this project investigated the growth-promoting effects of the ACC deaminase producing rhizobacteria *Variovorax paradoxus* 5C-2 and 3C-1 in a different species, broccoli (*Brassica oleracea*), under well watered and soil drying conditions in both pot and field trials. Drought and rhizobacterial impacts varied between trials. Seedling shoot fresh weight (SFW), root fresh weight (RFW) and leaf area (LA) increased by 50%, 42% and 43% respectively, when treated with *V. paradoxus* 5C-2 seven days after planting compared to the control. Conversely, when treated at seeding, SFW, RFW and LA were significantly decreased. As expected, drought treatment significantly reduced shoot dry weight of *Brassica oleracea* plants grown in pots in the greenhouse, but there was no effect of 5C-2 treatment during the drought period, unlike previous research. Field trials under commercial conditions indicated no effect of drought or rhizobacterial treatment with *V. paradoxus* on head weight. Thus rhizobacterial stimulation of early vegetative growth did not increase marketable yield.

Keywords — ethylene, ACCd, Variovorax paradoxus 5C-2 and 3C-1, drought, abscisic acid

Word count: 9019

List of Abbreviations:

5C-2: *Variovorax paradoxus* 5C-2

3C-1: *Variovorax paradoxus* 3C-1

ABA: abscisic acid

PGPR: plant growth-promoting rhizobacteria

ACC: 1-aminocyclopropane-1-carboxylate

ACCd: 1-aminocyclopropane-1-carboxylate deaminase

SFW: shoot fresh weight

SDW: shoot dry weight

RFW: root fresh weight

RDW: root dry weight

DAP: days after planting

WW: well watered

INTRODUCTION

General Background

One of the greatest global challenges facing the human race over the next 40 years is how to feed its growing population, which is expected to hit 9 billion by 2050, using less water and fewer resources, whilst land is progressively less available. Today, over 1 billion people are hungry and approximately 12.5% of the global population are undernourished (FAO, 2013). The UN Climate Change Conference in November 2012 thus posed the need for food production worldwide to increase by 60-70% of current production levels to attain food security (UN, 27 November 2012) and accommodate the needs of a (predicted) wealthier population who desire a more diverse range of high-quality food (Foresight, 2011).

The current demand, production and distribution of food globally does not form an indefectible model due to the multidiciplinary structure of the current food system (Foresight, 2011). A large proportion of the problems leading to this failing system are centralised around socio-political and economic issues (Godfray, et al., 2010), however, the effects of the fluctuating climate are also becoming more apparent and are starting to cause farmers extensive problems worldwide (Foresight, 2011).

Water-limited environments

The agriculture system currently uses about 70% of the global total of extracted fresh water and about 35% of global land use (Alexandratos & Bruinsma, June 2012). Although the majority of arable land worldwide is rain-fed, irrigation in agriculture is immensley important as it sustains roughly 44% of crop production (Alexandratos & Bruinsma, 2012). Whilst irrigation is essential in arid regions which are unsuitable for agriculture, in other regions, particularly in developing countries, it is used to supplement rain-fed crop under times of stress, such as dry seasons (Alexandratos & Bruinsma, 2012).

Water is becoming an increasingly limited resource as a result of increased demands, which have left water stores and groundwater levels depleted as they have been over-exploited in many areas (Siebert, et al., 2010). Climate fluctuation is creating a high level of unpredictability each year in weather patterns globally. Arable farming practices are becoming more unstable as farmers struggle to adapt to the changing weather patterns worldwide, which may lead to more or less rainfall in a year (Gregory, et al., 2005).

Here then lies the challenge for crop scientists and farmers alike, to find solutions for the intensification of arable land, to produce high quality,

nutritious and cheap food, using less water and fewer resources in a sustainable manner.

Plant responses to water-deficit

Water deficit within a plant occurs when the rate of transpiration is greater than water uptake (Bray, 1997). In their natural environments, plants may be subjected to short term (a few hours) or long term (gradual decrease in water availability over weeks) drought stress within their lifecycle (Chaves, et al., 2003).

Plant drought response to water-deficit causes reductions in crop growth and yield arising from changes in:

(i) Physiological properties, such as change in osmotic potential; which is a highly drought sensitive physiological process reliant on cell turgor pressure (Green & Cummins, 1974), reduction in leaf water potential, and a decrease in stomatal conductance leading to reduced net photosynthesis (Lisar, et al., 2012; Farooq, et al., 2009).

(ii) Molecular properties, such as increased stress responsive gene expression (ABA biosynthetic genes) and the synthesis of proteins like dehydrins, which play a protective role during cell dehydration (Hanin, et al., 2011).

(iii) Biochemical properties, by reductions in efficiency of Rubisco (a key enzyme in carbon metabolism within the leaves) and photochemical processes (Lisar, et al., 2012).

In response to long term stress, plants may shorten their lifecycle to avoid dehydration, and optimise their resource gain through acclimation to their new environment. For example, plants will close their stomata, shed leaves, and decrease leaf growth to reduce water loss, and invest carbon into the roots to maximise water uptake (Chaves, et al., 2003). Under short-term and rapid drought stress, plants react by minimising their water loss through stomatal closure (Zhang, et al., 2006), and change their metabolic pathways to avoid dehydration (Chaves, et al., 2003). For example, plants change the osmotic potential within the cells to favour water uptake (Bray, 1997).

Drought stress often occurs in conjunction with numerous other stresses, such as high temperature and salinity, which all induce water-saving responses. Yet these whole-plant mechanisms that may effectuate resistance to water deficit are largely dependent on the species and genotype of the plant, and the time-span in which the plant receives the stress at its different stages of development (Bray, 1997).

Water use efficiency

The water use efficiency (WUE) of a plant can be determined by the yield produced per unit of water used, and this varies greatly between species and genotypes (Blum, 2009). There have been significant advances in the understanding of plant physiological, biochemical and molecular responses to drought stress and selective breeding has favoured beneficial water use traits, however, there is still a large 'yield gap' where the optimal yield is not reached due to environmental stresses (Cattivelli, et al., 2008). With water becoming a more limited resource, increasing the 'crop per drop' is an important goal for agronomic research, whether this be through genetic manipulation, selective breeding or manipulation of the rhizosphere microbiome to influence hormone biosynthesis within crop plants (Wilkinson, et al., 2012; Blum, 2009; Cattivelli, et al., 2008).

Plant hormones

Over recent years, plant research has been focused toward hormones, as a greater understanding in this area permits the development of novel techniques for managing field crops, or for the development of new beneficial genotypes (Wilkinson, et al., 2012). Plant hormones control cellular process within plants ranging from growth regulation to plant defence response. The five 'classical' plant hormones are: auxin, gibberellin, abscisic acid, ethylene and cytokinins, which all function to control various plant physiological processes (Kende & Zeevaart, 1997).

Abscisic acid has a critical role in drought stress response as it acts as an endogenous messenger to regulate plant water status (Tuteja, 2007). Due the intensive cross-talk between abscisic acid and the ethylene metabolic pathways, these hormones can be considered the most directly involved in drought stress response in plants (Borsani, et al., 2002; Rosado, et al., 2006; Farooq, et al., 2009).

Abscisic Acid

The endogenous plant hormone abscisic acid (ABA) plays a key role in the regulation of plant water status. When a plants encounter drought stress they trigger a network of long-distance signaling events that induce genes which encode enzymes responsible for ABA biosynthesis (Rotchoudhury, et al., 2013). ABA is produced throughout the plant's lifetime to regulate processes from germination to shoot growth (Sharp & LeNoble, 2002a). However, it is primarily associated with stomatal closure in response to abiotic stress (Zhang, et al., 2006; Sharp, 2002b). ABA is involved in abiotic stress response within plants, from salt, cold, drought and wounding (Christmann, et al., 2006), and interacts with the plant hormone ethylene to adjust

developmental processes by restricting ethylene synthesis (Sharp & LeNoble, 2002a). However, ethylene also negatively regulates ABA biosynthesis creating a feedback loop (Christmann, et al., 2006). ABA is a multifunctional hormone with both promoting and inhibiting properties as a result of varied interactions with signalling pathways that are cell- or tissue-specific (Christmann, et al., 2006).

Ethylene

Despite the simplicity of its structure (C_2H_4), ethylene regulates many plant processes, but is most commonly known for its role in accelerating developmental processes such as fruit ripening or leaf abscission (Schaller, 2012). Ethylene is also involved in other stages of plant development from promoting seed germination and root hair development, to flower senescence (Wang, et al., 2002). A particular role of interest here is the response of ethylene to different biotic (pathogen attack), and in particular, abiotic (wounding, temperature, drought, ozone) stresses (van Loon, et al., 2006; Hays, et al., 2007; Wilkinson & Davies, 2010; Wang, et al., 2002). When a plant is subject to these stressful conditions ethylene biosynthesis is up-regulated, known as stress ethylene, to inhibit some plant functions as a defensive measure, including crop yield, root growth, and shoot/leaf expansion (Ables, 1972; Glick, 2005).

Ethylene is formed from S–adenosyl-methionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC). The first step in the biosynthetic pathway is the conversion of SAM into ACC, catalysed by the enzyme ACC synthase (Fig 1). ACC is then converted to ethylene by ACC oxidase (Bleecker & Kende, 2000). ACC synthase also produces 5'-methylthioadenosine (MTA) in the reaction which is used to synthesise new methionine for the beginning of the cycle, therefore ethylene biosynthesis can be maintained at high rates even if the source of free methionine is low (Bleecker & Kende, 2000). ACC may also be converted to 1-malonyl-ACC (MACC), catalysed by *N*-malonyl transferase (which is widely present in plant tissues), and/or converted into γ-glutamyl-ACC (GACC) via γ-glutamyltranspeptidase, as a potential mechanism to downregulate ethylene levels (Peiser & Yang, 1998; McDonnell, et al., 2009). These conjugates correspond with ethylene production rates dependent on the developmental stage of the plant, primarily ripening, or external influence (excision, temperature stresses or exogenous application of ethylene) (Liu, et al., 1985; Machackove, et al., 1989).

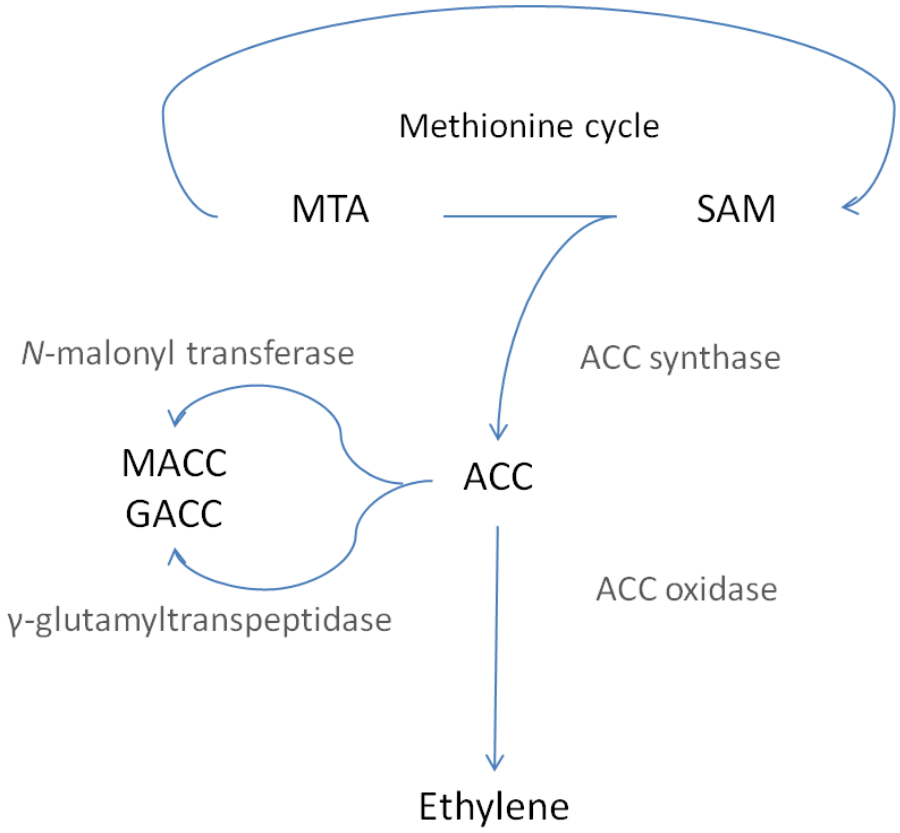


Figure 1. A simplified version of the ethylene biosynthetic pathway. Adapted from (Vriezen, et al., 2003).

Plant Growth Promoting Rhizobacteria (PGPR)

The layer of soil surrounding plant roots, known as the rhizosphere, is a highly active area of root activity and metabolism which contains a unique population of microorganisms that is influenced by plant exudates (such as amino acids, fatty acids, sugars, plant growth regulators) which can be utilised by these microorganisms as nutrients (Saharan & Nehra, 2011; McNear Jr, 2013). The number of microorganisms colonising plants can reach cell densities much greater than the number of plant cells making the rhizosphere microbiome a complicated food web (Mendes, et al., 2013) The microorganisms living in the rhizosphere largely consist of plant growth promoting rhizobacteria (PGPR) (Saharan & Nehra, 2011; Lugtenberg & Kamilova, 2009), however, the microbial community also contains plant pathogenic microorganisms and opportunisitic human pathogenic bacteria (Mendes, et al., 2013). The interactions between these organisms are

complex and little understood, making the rhizosphere one of the most complex ecosystems on Earth (Mendes, et al., 2013).

PGPR have a variety of direct and indirect positive effects upon plant growth. Indirect effects are principally associated with the biological control of soil-borne plant diseases, which is more environmentally friendly than the use of pesticides (Saharan & Nehra, 2011). The direct effects of PGPR however, include use as:

(i) biofertilisers to assist in nutrient uptake by the plant or increase root development
(ii) rhizomediators using pollutant-degrading rhizobacteria
(iii) stress controllers through mediation of plant hormone status to manipulate root-shoot signalling and to mediate changes in root growth and architecture (Lugtenberg & Kamilova, 2009; Dodd, et al., 2010).

The use of rhizobacteria for biocontrol is the more commonly targeted area of research, as it is widely expressed that the use of PGPR that directly affect growth and development is difficult to reproduce in the field because of the influence biotic and abiotic factors which may negatively impact upon microorganism productivity due to competition and unfavourable conditions (Glick, 2005). However more recently, numerous studies have shown field success, in the maintenance or enhancement of yield under stress conditions, with involvement of the PGPR containing the enzyme ACC deaminase (Dey, et al., 2004).

ACC deaminse producing rhizobacteria

Bayliss et al. (1997) proposed that much of the ACC produced in the ethylene biosynthetic pathway is exuded by the seeds and roots along with the other small molecules present in root/seed exudates (Bayliss, et al., 1997). One mechanism by which PGPR promote growth is through the production of the enzyme ACC deaminase (ACCd). This enzyme cleaves the plant ethylene precursor ACC to yield ammonia and α-ketobutyrate, thereby limiting the biosynthesis of ethylene levels (Glick, 2005; Lugtenberg & Kamilova, 2009).

Some PGPR synthesize and secrete indole-3-acetic acid, (IAA) which gets absorbed onto the seed or root surface, some of which is taken up by plants and can stimulate plant cell proliferation and elongation (Saleem, et al., 2007). IAA also stimulates ACC synthase to convert SAM into ACC (Glick, et al., 2007). ACC is exuded from the plant root/seed, taken up by the rhizobacteria and hydrolysed by ACCd (ACCd is not a secreted enzyme and remains in the cytoplasm of the rhizobacteria) (Glick, 2005; Saleem, et al., 2007). The uptake of ACC by the PGPR alters the equilibrium between plant internal and rhizospheric ACC levels, therefore the plant produces and

exudes more ACC to compensate, leading to overall reduced levels of ACC within the plant root/seed whilst providing microorganisms with a source of fixed nitrogen (in the form of ammonia) and carbon (Figure 2) (Glick, et al., 1998; Belimov, et al., 2005). Therefore, under the production of 'stress ethylene', the PGPR act as a sink for ACC, limiting the production of ethylene within the plant (Glick, 2005). Consequently, PGPR containing ACCd, should, when bound to roots/seed coats, enhance root and shoot growth as a consequence of reduced ethylene levels within the plant, regardless of external stress factors (Glick, et al., 1998; Glick, 2005).

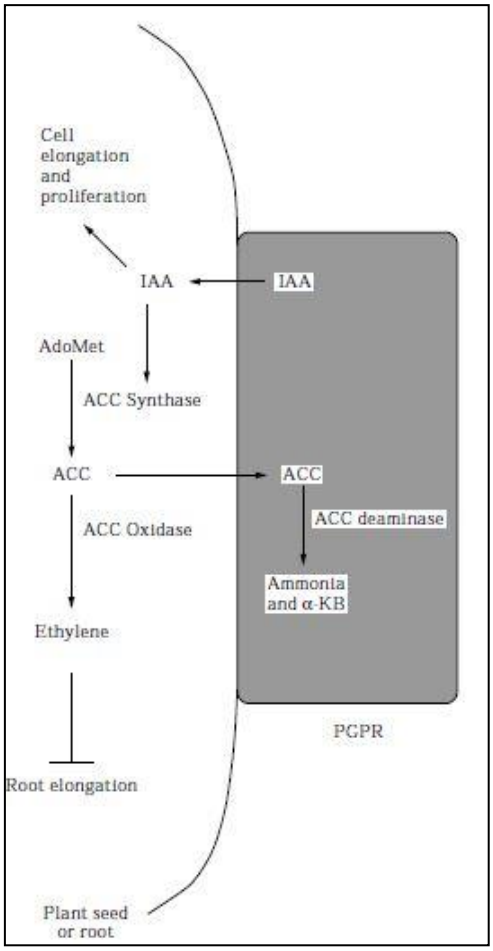


Figure 2. Taken from (Glick, et al., 1998). A schematic diagram showing how root ethylene concentration decreases when ACCd producing PGPR are in vicinty of the root. Key: IAA: indole-3-acetic acid, ACC: 1-aminocyclopropane, α-KB: α-ketobutyrate

Variovorax paradoxus 5C-2 and 3C-1

Variovorax paradoxus 5C-2 and 3C-1 (previously known as 2C-1) are two of many known species of ACCd producing PGPR . *V. paradoxus* 5C-2 and 3C-1 were first isolated from the root zone of Indian mustard (*Brassica juncea* L. Czern), and were found to promote its root growth in the presence or absence of toxic Cadmium concentrations (Belimov, et al., 2005). Both 5C-2 and 3C-1 were able to use ACC as a sole source of carbon, suggesting their production of ACC deaminase (Belimov, et al., 2005).

Chen et al., (2013) explicitly linked the effects of *V. paradoxus 5C-2* and the consequential change in foliar ethylene production to changes in growth and development as a result of ACCd action. Soil inoculation of *V. paradoxus* 5C-2 increased leaf area and shoot biomass of both wild type and an ethylene-overproducing mutant of *Arabidopsis thaliana* species, but not in ethylene-insensitive mutants, indicating an ethylene-dependent pathway, which followed the model proposed by Glick et al., (1998)(Chen, et al., 2013). Although prior to this, Mayak et al., (2004) had already linked ACCd production (by the rhizobacterium *A. piechaudii* AEV8) to whole plant ethylene production in tomato and pepper seedlings in response to water deficit (Mayak, et al., 2004).

The growth-promoting effects of *V. paradoxus* 5C-2 have been shown in a number of species. Jiang et al. (2012) found increased shoot and root biomass (20% and 15% respectvely) in pea with 5C-2 innoculation. Similar results from Belimov et al. 2009) showed this rhizobium to increase shoot and root dry weights of pea plants in drying soil, and also increased their water use efficiency (WUE) by almost 50% compared to the untreated controls (Belimov et al. 2009). Furthermore, under deficit irrigation, maize growth was promoted with 5C-2 inoculation which is also attributed to ACCd production by this rhizobacterium, which thus limited ethylene biosynthesis under water stress (Dodd, et al., 2009).

V. paradoxus 5C-2 has also been shown to influence ABA relations in pea (Belimov, et al., 2009; Jiang, et al., 2012; Chen, et al., 2013) but had no effect on systemic ABA signalling in maize under well-watered or soil drying conditions (Dodd, et al., 2009). Systemic effects of 5C-2 inoculation in pea plants included a decrease in xylem ABA concentration, as much of the lower shoot xylem ABA was released into the phloem (Jiang, et al., 2012). In the same experiment, root ABA biosynthesis and accumulation were also drecreased by 46% and 55% respectively. However, under soil drying there was an increase in induced xylem ABA concentration and a decrease in induced xylem ACC concentration in pea plants (Belimov, et al., 2009).

The contrasting results of the current research conducted with 5C-2, are most likely explained through species dependent differences in the role of ethylene in the different developmental stages. Jiang et al., (2012) studied the effects of 5C-2 on ABA relations within the plant, but there was no further investigation into the interactive effects of the 5C-2, ABA and soil drying. Further reseach looking at ACC, ethylene and ABA flow throughout the whole plant under soil drying would be advantageous, as there is current contention as to whether soil drying increases leaf ethylene evolution (Morgan, et al., 1990; Belimov, et al., 2009). Belimov et al., (2009) did not measure ABA in drying soil, but Morgan et al., (1990) found between-species differences in ethylene production in repsonse to water deficit, which explained this as an artefact of rapid impostion of water stress (Morgan, et al., 1990). Sobieh (2004) however, suggests that soil-drying can influence ethylene evolution and thus modfy leaf growth (Sobeih, et al., 2004). As there is contention of ethylene evolution in leaf production in repsonse to water deficit, further work must be conducted using a greater variety of species. Hormone-flow modelling should be analysed to explain how ethylene and its precursors are locally and systemically influence through the plant.

Although shoot and root biomas as well as water use efficiency have been shown to increase with 5C-2 innoculation in a number of species (Belimov, et al., 2009; Chen, et al., 2013; Dodd, et al., 2009; Jiang, et al., 2012), investigation into productivity of inoculated plants under field study has yet to be fully approached in the published literature.

AIMS AND OBJECTIVES

Improving crop growth and water use efficiency through hormone manipulation using rhizobacteria, is a cheap and environmentally friendly method of improving the sustainability of our agricultural systems.

So far, research has shown conflicting results of the effects of *V. paradoxus* 5C-2 between different crop plants (Belimov, et al., 2009; Dodd, et al., 2009; Jiang, et al., 2012). Further studies using different genera must be conducted to determine whether the plant growth-promoting effects of 5C-2 are largely universal, or whether the hormone interactions are more species dependent.

The rhizobacteria *V. paradoxus* 5C-2 and 3C-1 were originally sourced from *Brassica juncea (*Indian mustard), making it likely that they would thrive on other brassica crops. One of the priority objectives of the Horticultural Development Company (HDC), as indicated in the 'Brassica Growers Association Research and Development Strategy (2011-2013)' under the objective of increasing returns on investment through efficient use of resources, is to understand how to grow Brassicas with drought tolerance, potentially through use of microbes in the soil (HDC, 2011).

Consequently, this project investigated whether the PGPR *V. paradoxus* 5C-2 and 3C-1 could promote growth of *Brassica oleracea* var. Kabuki F1 (calabrese) under well watered and drought stress conditions. Calabrese was the brassica crop chosen for this study because it grows during the summer months and has excellent nutritional benefits (USDA, 2009). There are also links to high consumption and reduced risk of prostate cancer with this crop and other brassica species (Kristal & Lampe, 2002) making it an highly valued food.

METHODS AND MATERIALS

Preparation of bacteria for inoculation

To prepare *V. paradoxus* 5C-2 and 3C-1 inocula, the rhizobacteria were plated onto Tryptic Soy Agar (TSA) from a frozen stock and grown at 30^{o}C in an incubator. Subcultures were then taken from these plates and left for 48 hours at 30^{o}C in the incubator. Tryptic Soy Broth (TSB) was prepared as a medium for bacterial growth for inoculation of seeds and seedlings. The broth was autoclaved in 500ml and 250ml conical flasks which were filled to 75% of their maximum volume to ensure sufficient air for aerobic respiration by the bacteria once they were capped with foil, which was used to eliminate the risk of contamination and spillage.

Under sterile conditions, 3 plates of *V. paradoxus* 5C-2 or 3C-1 were added per 800ml of sterile TSB broth. The inoculated broth was then left for 15 hours in a shaker incubator at 250rpm and 22^{o}C. To remove the broth from the bacteria, the solution was centrifuged at 22^{o}C and 4000g for 20 minutes. The resulting supernatant was decanted and the pellet resuspended in small volumes of deionised water. The suspension was then poured into 50ml centrifuge tubes. These tubes were then centrifuged at 22^{o}C and 4000g for 20 minutes. The centrifugation process was repeated as required to remove all TSB from the inoculums to obtain approximately 250ml of concentrated bacterial solution per 6 litres of inoculated TSB. From the 50ml centrifuge tubes the bacteria was combined into a 250ml conical flask to make the final solution.

An optical density of the concentrated solution was determined using a spectrophotometer set at 570nm (with a reference of deionised water) using a 1 in 10 dilution with deionised water which was then multiplied tenfold to establish the true density. Seedling modules at Fountain Plants (Lincs.) were inoculated with an OD of 0.2, and the plants at Lancaster were inoculated with an OD of 0.05 per litre of soil. E.g. add 2ml of concentrated inoculum at OD 0.05 for 1 litre of soil, add 8ml for 4 litres of soil.

Two different ACCd producing rhizobacteria were used in these experiments because 5C-2 cannot be easily commercialised.

Glasshouse: Seedling trials (Lancaster University)

Seedling trays holding 77 plants in 35ml plugs, filled with Levington M3 (Levington, UK) were used to assess the initial effects of *V. paradoxus* 5C-2 and 3C-1 on calabrese (*Brassica oleracea* var. Kabuki F1) obtained from

Moles Seeds (U.K) Ltd.). Calabrese were inoculated using 5C-2 and 3C-1 at seeding and 7 days after seeding to look at whether the rhizobacteria had different effects on the growth of the plant and whether this differed if the plants were inoculated at different developmental stages.

A further seedling trial was set up to study the effects of 5C-2 on calabrese. Calabrese was germinated for seven days in seedling flats which were cut in half prior to placing in propagator trays (two halves per tray). Each tray received 1L of water at seeding, and thereafter 1L or 500ml by visual assessment of how much water remained in the trays. The trays were rotated in a circular manner daily in the glasshouse to eliminate any between-tray effects. Seven days after planting (DAP) one of the halved seedling flats from each tray was moved into their own trays for treatment. From each propagator tray one half was inoculated with 5C-2 and the other half remained as a control (Appendix A).

Glasshouse: Pot trials (Lancaster University)

Calabrese (*Brassica oleracea* var. Kabuki F1) were seeded into three trays holding 84 plants in 35ml plugs filled with an organic substrate (Levington's M3, Levington, UK) on 19th April 2013. The glasshouse was set at a relatively controlled day time temperature of 22^{o}C and night time temperature of 17^{o}C with daylight hours between 8am and 10pm. However, the ability to control the temperature of the glasshouse when the ambient temperature exceeded 22^{o}C was limited. In late May through to July, day time temperatures frequently exceed 25^{o}C (max. 36.8^{o}C).

The seedlings were grown for 7 days in propagator trays and were kept well watered by maintaining a layer of water at the base of the trays. Twenty four days after planting (DAP), 100 calabrese seedlings (selected from the middle of each of the trays to assure uniformity) were transplanted into 4L pots containing John Innes no.2. (J. Arthur Bowers, Lincoln, UK). Half of the plants were inoculated with *Variovorax paradoxus* 5C-2 at 31DAP with an OD of 0.05 per litre of soil (Appendix B).

Root colonisation assay

To assess root colonisation by 5C-2, and to confirm there was no cross-contamination between the pots, root colonisation was measured 7 days after inoculation (38 DAP) and 43DAP.

V. paradoxus 5C-2 is resistant to antibiotics kanamycin and rifampin. Consequently, to asses root colonisation, kanamycin and rifampin were supplemented to tryptic soy agar (TSA) at concentrations of 30µg ml^{-1}, 20µg ml^{-1} respectively to eliminate growth of most other soil microorganisms. A

further antifungal compound, nystatin, was added at a concentration of 40µg ml^{-1}. The agar was then poured into plates (2 per plant).

To collect 5C-2 from the rhizosphere, 0.5g of roots were collected from the top of the root system directly beneath the shoot, and ground in a pestle and mortar with 1ml of distilled water under sterile conditions. Four serial dilutions from the resulting solution were created. Under sterile conditions, 9µl of distilled water was added to each of four microcentrifuge tubes. To the first tube, 1µl of the root solution was added and the solution mixed. From this first tube, 1µl of solution was obtained and added to the second tube, creating 1:10 serial dilutions up to the fourth microcentrifuge tube, which was at a concentration of 10^{-4}.

The plates were marked in half and labelled accordingly (10^{-1} to 10^{-4}) with two concentrations per plate. 1ml of each respective solution was carefully pipetted across half of the plate. The plates were then incubated at 30°C for 96 hours. After the incubation period the numbers of colonies per dilution per plate were counted and the colony forming units were established using the equation:

Colony forming units = number of non-overlapping colonies *x* the inverse of the dilution for 1ml

Drought treatment

The day after the calabrese seedlings were transplanted into the 4L pots containing John Innes No. 2 (32 DAP), they were watered until run-through from the bottom of the pots, to ensure full saturation of the soil. Six hours later the pots were weighed to give drained capacity of each pot. Each morning the plants were weighed and watered back up to their individual well-watered weights.

Pre-drought (45 DAP), 24 plants were harvested (cut at the soil surface) to measure shoot fresh and dry weights and leaf area and ABA concentration of the second most fully expanded leaf using a ABA radioimmunoassay based on a previously described method (Quarrie, et al., 1988). Half of the remaining plants were subjected to a drought treatment of 50% of (well-watered) control evapotranspiration creating a 2 x 2 factorial experiment.

Plants were watered each morning for 14 days and soil moisture (moisture meter HH2 Delta-T Devices, Cambridge, UK), stomatal conductance (porometer AP4 Delta-T Devices, Cambridge, UK), leaf water potential and leaf osmotic potential (HR-33T Dewpoint microvoltmeter) measured periodically, 1 hour after watering. The seventh leaf from the base of the plant was used to measure stomatal conductance and to take leaf discs to measure water relations. A mid-drought harvest (52 DAP) was taken of three

plants from each treatment and the end-of-drought harvest was taken 58 DAP of 19 plants at which point foliar ethylene evolution was measured.

At 59 DAP the droughted plant were brought back up to their well-watered state. At this point plants were watered with Miracle-Grow® (according to the manufacturer's instructions) every 5 days. The plants were then grown through to heading and harvested at the same time, 87 DAP, when all of the plants had reached a commercially viable size, although some at this point had begun to flower.

Ethylene analysis

Leaf samples weighing 1g were collected at harvest from the most recent fully expanded leaf. The leaf section was contained in a bunged tube with some damp tissue for 1 hour under lamplight. Using a gas tight syringe (SGE 5ml gas tight syringe with removable needle, Sigma Aldrich), 4ml of gas was collected from the unopened tube and contained in gas-tight vials (5.9ml Exetainer®, Labco). The samples were analysed by gas chromatography (GC) using an Agilent 5973 *Network* Mass selective detector. Within a two-week period, 1ml of stored gas was injected into the GC machine using a SGE 1ml gas tight syringe with removable needle (Sigma Aldrich).

Commercial trials with Produce World

Fountain Plants: Inoculation

Calabrese (*Brassica* oleracea var. Steel) were machine seeded at Fountain Plants Limited (Boston, Lincs.) in seedling trays holding 345 plants with 14ml plug size on 12th April. Six litres of *V. paradoxus* 3C-1 were produced (see section x) but kept in TSB for ease of application to the plants. The inoculum was diluted to OD 0.2 at 570nm using tap water on site. Six trays of plants were treated with 3C-1, 3 were treated with TSB (to assess potential effects of the solution without bacteria) and a further 6 were left as a control. Two litres of diluted inoculum was watered onto each tray from a watering can with a rosette head. Control trays each received 2L of tap water in the same manner and 3 received the TSB. Each plug received approximately 5.8ml of liquid. The trays were left surrounded by other commercial trays to avoid any edge effects and/or contamination (Appendix C). These plants were treated with the insecticide DURSBAN® WG at the recommended rate alongside the other Brassicas at Fountain Plants Ltd.

Field trial: Produce World (Lincs.)

Seven week old seedlings (48 DAP) from the treated trays at Fountain Plants Ltd. were transplanted into a field at Produce World (Boston, Lincs.). Eight plots were created of 15m^2 which each held 90 plants at 50cm spacing

aligned with ploughing marks to follow commercial planting conditions (Appendix D). There were 3 plots of control and 3C-1 inoculated plants and 2 plots of broth treated plants randomised across the field (Fig. 3) .The plants were selected from the middle of each of the trays in aiming to maintain uniformity in the seedlings. At harvest, plots 6, 7 and 8 were ignored because there were very few harvestable heads.

This field trial was grown under commercial (rain fed) conditions and was harvested 114DAP. Mid-way through the season the crop was hand weeded, covered and sprayed for weeds and pests. One tray of control plants and one tray of inoculated plants were brought back to Lancaster University to measure leaf area and fresh and dry, root and shoot weight, at 51 DAP.

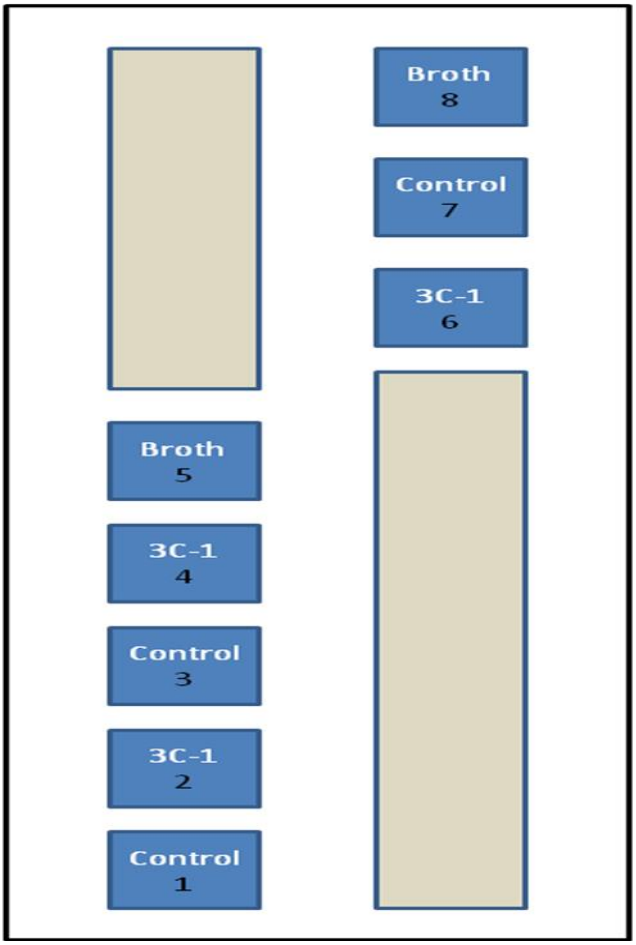


Figure 3. Diagram to depict the approximate layout of the field site at Produce World (Lincs). Each plot contained 90 plants with equal 50cm spacing and a gap of 2m between each plot, which are numbered and the treatment identified. The grey strips show the proximal layout of other brassica crops grown in the same research field.

Field trial: Lee Farm (Lancs.)

The remaining plants from the trays brought back from Fountain Plants Ltd were used in a second field trial at Lee Farm (Bilsborrow, Lancs.) in association with Myerscough College (Lancs.). The objective of this field trial was to determine the effects of *V. paradoxus* 3C-1 on calabrese growth and water status under drought conditions. The field site (13 x 7 m) was covered by a semi-transparent plastic (poly-tunnel) to exclude rain. The site was rotavated to a depth of 30cm and levelled. The soil had a mineral content that was predominantly sandy (93% as measured in 2008) with silt (2.8%) and clay (4.2%).

An irrigation system was installed and arranged into four alternate wet and dry beds which were 1m apart, and the plants were transplanted 80 DAP with 50cm^2 spacing as depicted in Fig. 4a (Appendix E). The seedlings were selected from the middle of the trays to maintain uniformity. Following transplantation fertiliser was spread over the bed at levels recommended by PLANET nutrient management (200kg/ha N, 225kg/h K_2O) (ADAS, 2010). Carpet underlay was also placed around the base of the plants to prevent female cabbage root flies laying eggs around the base of the plant (Fig. 4b) (RHS, 2013).

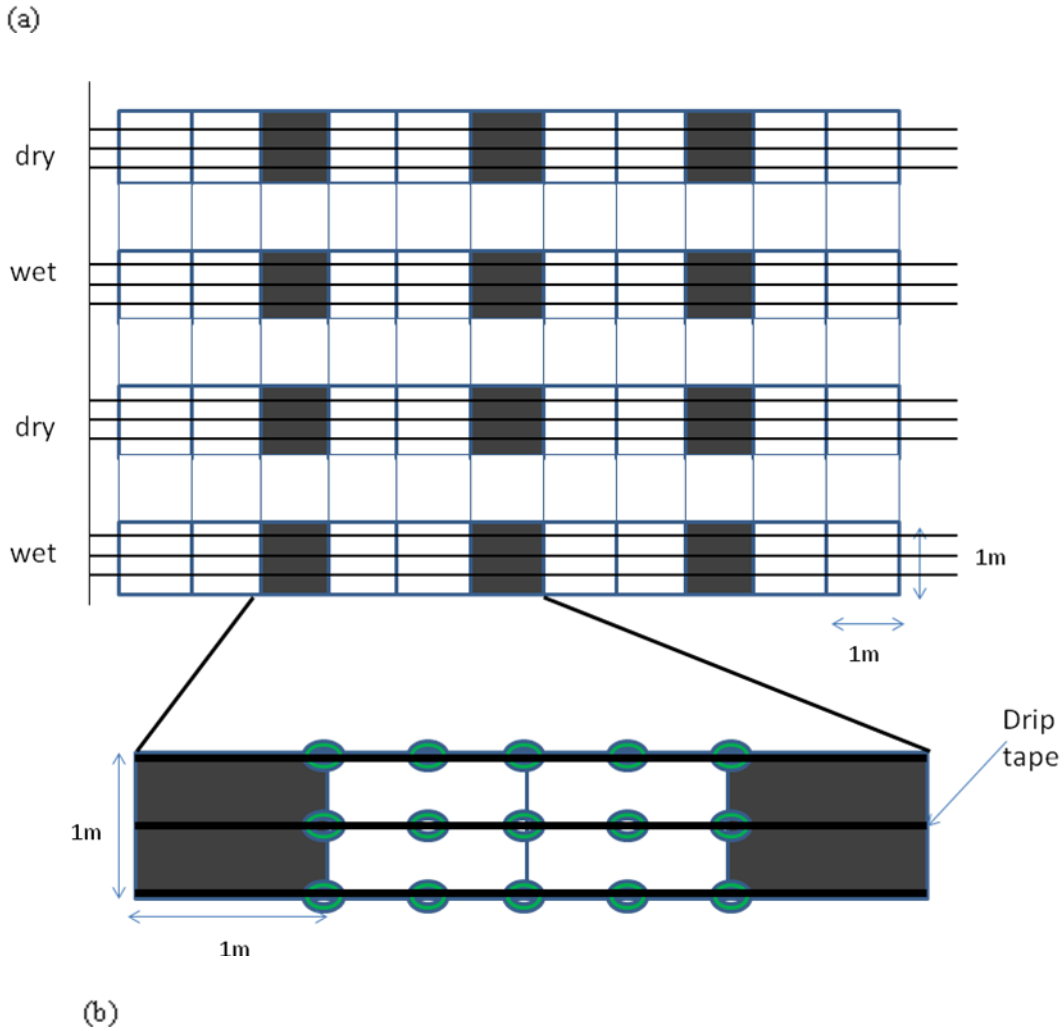
For ten days after transplanting, all plants were kept well watered by the irrigation system, receiving water for thirty minutes twice a day (6am & 6pm) to enable good establishment within the soil. On the eleventh day, half of the irrigation pipes were turned off to start the drought treatment to half of the plants.

Soil moisture and stomatal conductance were measured at 72 DAP and 85 DAP. At 99DAP and 112DAP a third and fourth visits soil moisture and stomatal conductance measurements were again taken, but also this time leaf sample were snap-frozen in liquid nitrogen for ABA analysis. The calabrese did not reach heading before the final harvest which was taken 118 DAP. The three middle plants from each plot were sampled to eliminate edge effects (Fig. 4a).

Statistical analyses

Unless otherwise stated, all data with multiple variables was analysed using either a one-way ANOVA or two-way ANOVA followed by the *post-hoc* Tukey HSD. Independent samples t-tests were run where appropriate. In some cases non parametric tests (Kruskall-Wallis, Welch's ANOVA with relevant *post-hoc* tests; Dunn's procedure with a Bonferroni correction, Games-Howell etc.) were used due to violations in normality or outliers within the data where transformation was not successful, in which case this will be identified within the text. Statistical significance was accepted at the 0.05 level and analysed using the statistical software package SPSS (version 20).

Figure 4. (a) The layout of the polytunnel at Myerscough College. The Grey areas are fallow. The green circles indicate the spacing of each plant: 0cm, 50cm and 100cm down, then similarly spaced across a 2m length, giving a total of 240 plants. (b) Carpet underlay placed around the base of each plant to prevent cabbage root fly



RESULTS

Seedling trials

Seedlings were inoculated either at seeding or 7DAP and each trial ran for 21 days at which point plants were harvested. Soil inoculation with 5C-2, 7DAP, significantly increased SFW ($p = 0.004$) (Fig. 5), SDW ($p = .012$) and LA ($p = 0.004$) by 50%, 42% and 43% respectively, compared to control plants (Appendix F). However, seedlings that were treated at seeding with 5C-2 had significantly lower SFW, SDW and LA ($p = 0.001$, $p = 0.014$, $p = 0.01$ respectively) (Appendix B). Soil inoculation with 3C-1, both at seeding and 7DAP significantly decreased SFW and LA ($p < 0.001$).

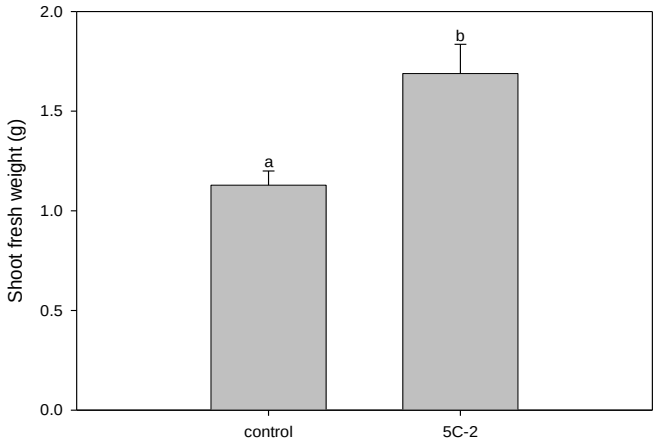


Figure 5. Shoot fresh weight of inoculated and uninoculated broccoli seedlings. Data are means ± SE of replicates (n = 19 control, n = 14 5C-2)

Inoculated seedlings (brought back from Fountain Plants) had significantly increased SFW, RFW and LA ($p < 0.001$), but not SDW or RDW ($p > 0.1$) compared to uninoculated plants (Table 1).

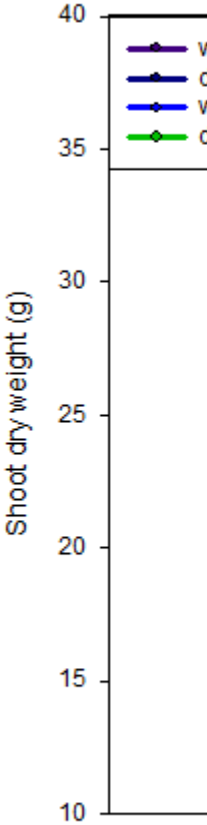
Table 1. Effects of *V. paradoxus* 3C-1 inoculation on seedling growth (47 DAP). Data are means ± SE of n replicates, with significant ($P < 0.05$) differences asterisked.

Treatment	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Leaf area (cm^2)
Control	1.27 (±0.02)*	0.21 (±0.01)	0.63 (±0.02)*	0.06 (±0.001)	21.31 (±0.41)*
3C-1	1.5 (±0.44)*	0.23 (±0.01)	0.77 (±0.03)*	0.07 (±0.002)	25.12 (±0.59)*

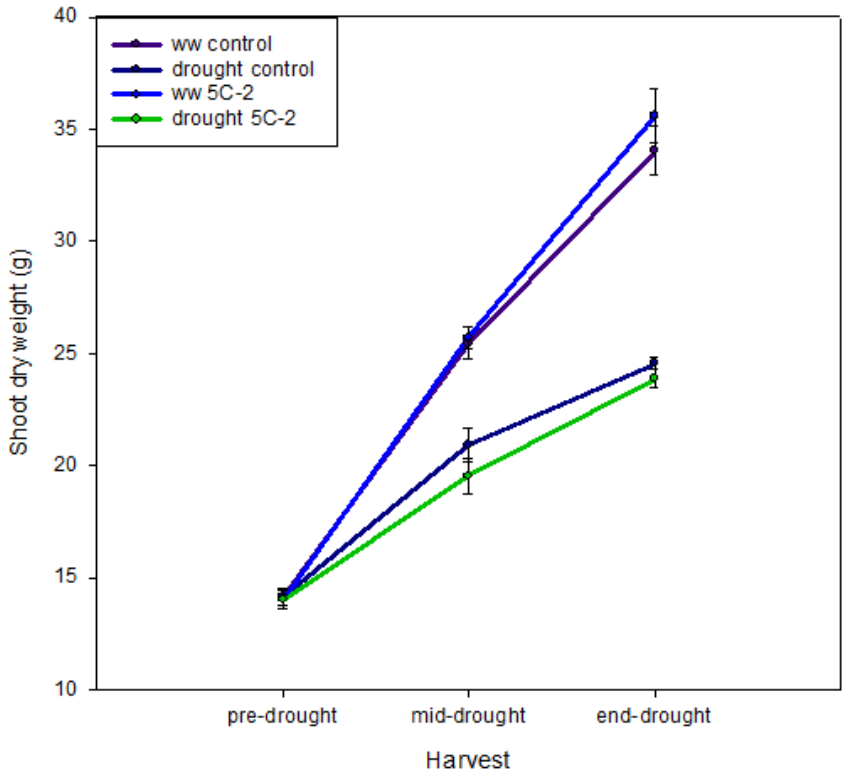
Pot trial

Rhizobacterial colonisation of the root system was measured five days after inoculation and at the end-drought harvest. The bacterial concentration increased from an average of 20^{-4} cells g^{-1} soil to an average of 10^{-5} cells g^{-1} soil over this period.

Drought treatment significantly decreased SDW ($p < 0.001$) compared to the control in the mid-drought and end-drought harvests (Fig. 6a). There was no significant impact of 5C-2 ($p = 0.461$). Pre-drought treatment of 5C-2 had no effect on SDW (measured the day before soil drying began). SFW and LA were also measured and followed the same patterns of significance (data not shown). By the final harvest, the well watered 5C-2 treated plants had a significantly increased SDW ($p = 0.039$) compared to the droughted 5C-2 treated plants (data not shown). Two-way ANOVA on this harvest showed the difference to be due to water treatment ($p = 0.015$) and not 5C-2 ($p > 0.05$). There was no significant difference between the uninoculated plants, well watered or drought treated. At the final harvest, head fresh and dry weights were also measured. There was no significant difference ($p > 0.05$) between the treatments (Fig. 6b).



(a)



(b)

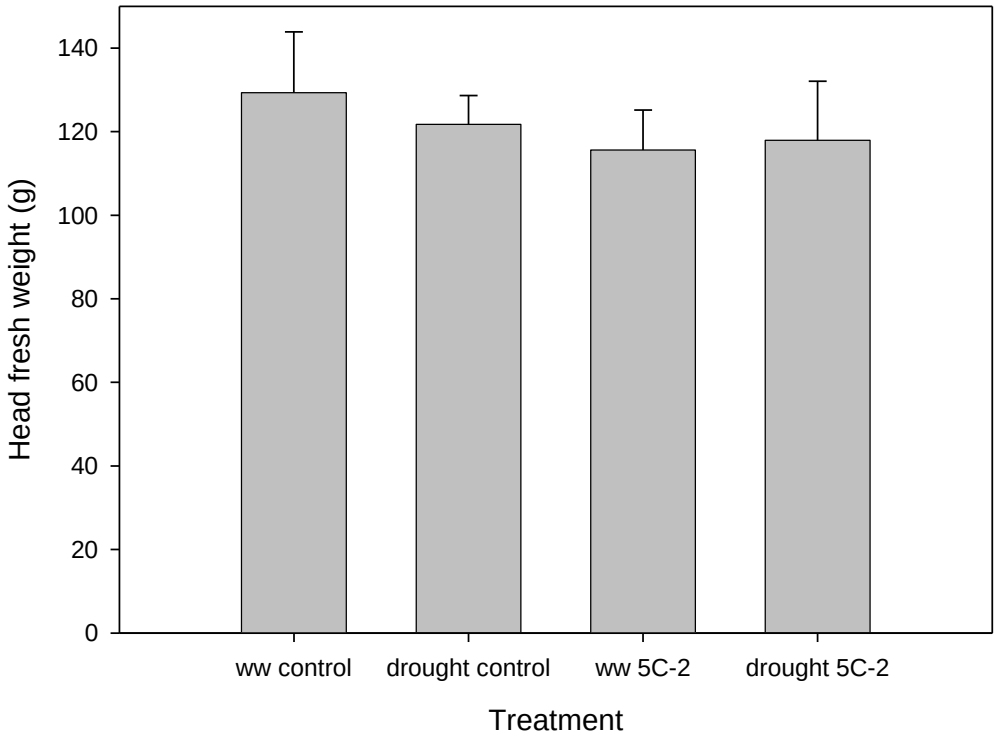


Figure 6. (a) Three harvests were taken throughout the drought treatment. Change in SDW over time is shown across the line graph with error bars for each plot. There were significant differences ($p < 0.05$) in SDW between the well watered and drought treatments at both the mid-drought and end-drought harvests but no impact of 3C-1. (b) Head fresh weight was measure at the final harvest. There was no significant difference ($p > 0.05$) between the treatments.

Levels of soil moisture measured periodically during the pot trial showed the droughted plants had lower soil moisture than the well watered plants throughout the period of drought treatment ($p < 0.05$).

Stomatal conductance varied throughout the pot trial (Fig. 7). There was no significant difference ($p = 0.46$) in stomatal conductance in the pre-drought measurements (4th June) between treatments. Mid-drought (9th June) stomatal conductance was significantly different between the well watered and drought treatments. Two-way ANOVA showed no interactive effect of water or 5C-2 treatment ($p = 0.57$), but drought treatment and 5C-2 had significant main effects ($p < 0.001$, $p = 0.013$ respectively), both independently reducing stomatal conductance. There were also significant

differences in stomatal conductance between the treatments at the end-drought (15th June) harvest. There was no interactive effect of the two treatments ($p = 0.272$) and no effect of 5C-2 ($p = 0.615$). However, water treatment did significantly decrease ($p < 0.001$) stomatal conductance at the end of the drought period.

There was a significant increase ($p = 0.001$) in stomatal conductance of the droughted plants after re-watering, (22nd June, although this was measured 5 days after re-watering to ensure all plants were in well watered soil). There was no significant effect of bacterial treatment ($p = 0.49$).

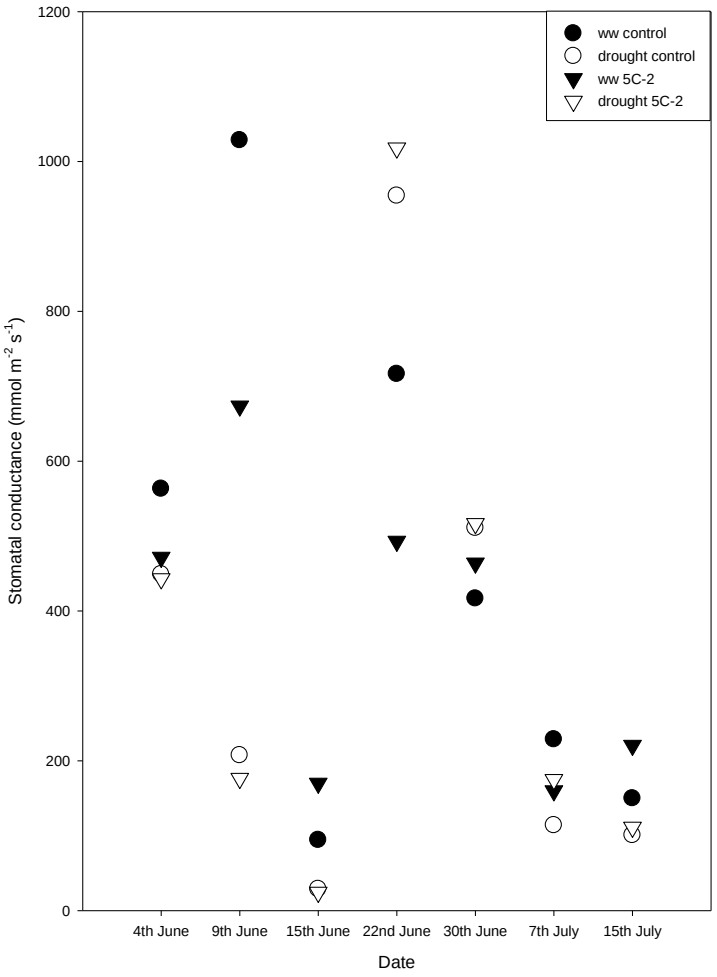
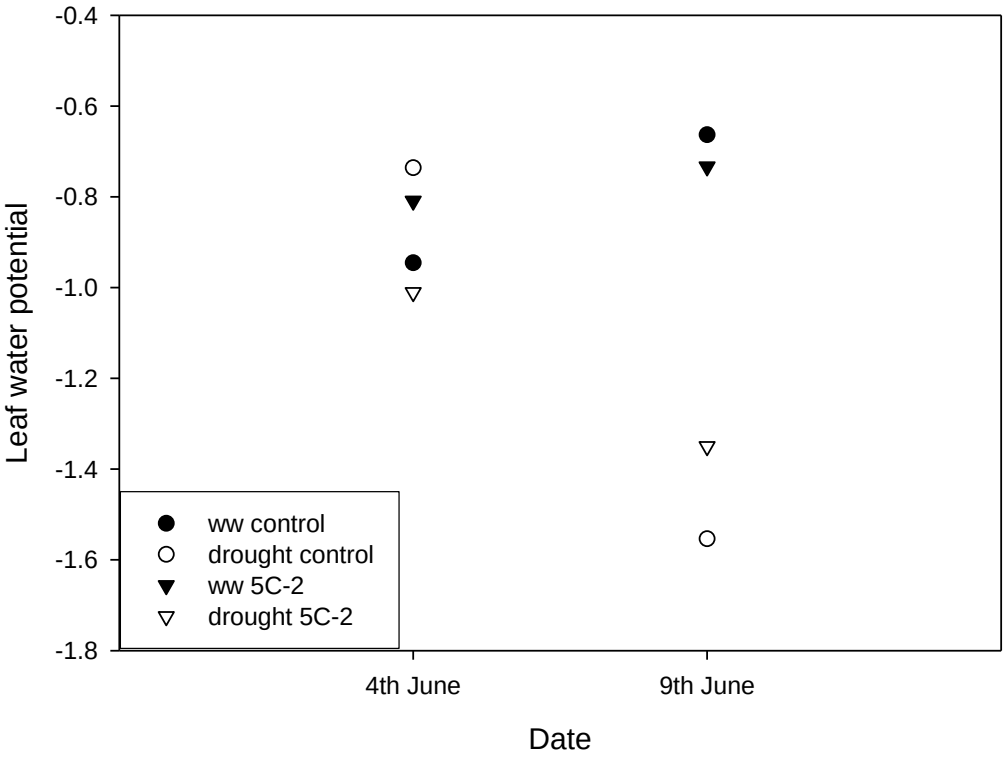


Figure 7. Stomatal conductance in the pot trial. Stomatal conductance significantly decreased ($p < 0.05$) in the droughted plants between 4th June and 15th June. Data are means of n replicates, error bars omitted for clarity.

There was no significant effect ($p > 0.05$) of rhizobacterial inoculation in pre-drought (4th June) leaf water potential, Ψ_{leaf} (Fig, 8a and 8b). There was no significant difference ($p > 0.05$) in Ψ_{leaf} between the treatments (one-way ANOVA). However, two-way ANOVA showed drought treatment to significantly lower Ψ_{leaf} and osmotic potentials ($p = 0.002$, $p = 0.009$ respectively). There was no effect of 5C-2 ($p > 0.05$).

(a)



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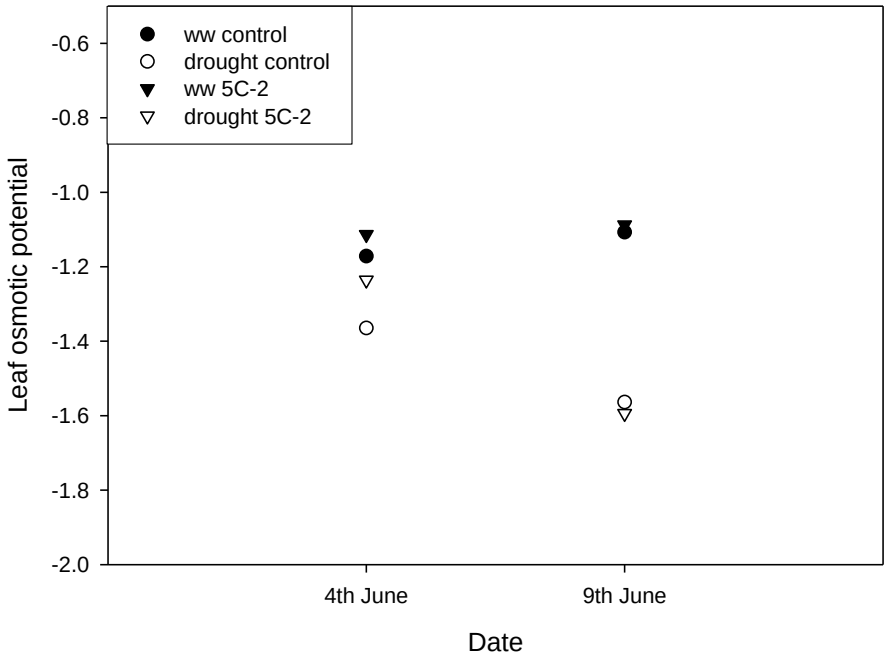


Figure 8. Leaf water potential (a) and leaf osmotic potential (b) were measured pre-, mid- and end-drought for each of the treatments. Data are means of 5 replicated. Error bars were omitted for clarity. .Pre-drought (4th June) there was no significant effect ($p > 0.05$) of any of the treatments on leaf water potential. Watering treatment did significantly affect ($p < 0.05$) leaf water potential mid- and end-drought.

Figure 9a shows the end-drought mean values and figure 9b shows the final harvest mean values of foliar ethylene analysis. Two-way ANOVA showed no interactive effects of the treatments ($p = 0.385$), no significant effect of 5C-2 inoculation ($p = 0.177$), and no significant effect of water treatment ($p = 0.057$) on the end-drought harvest. However, there were few repeats (drought control n = 1) which will have influenced the statistical analyses. There is a tendency for drought to increase ethylene evolution as the p-values of water treatment neared 0.05.

There was also no significant impact of water treatment (p = 0.935) or 5C-2 inoculation (p = 0.07) in ethylene levels at the final harvest, although p-value of 5C-2 inoculation did near significance (Fig. 9b). Also, noting that the overall mean leaf ethylene values of the second harvest are much higher (Fig. 9a & 9b) than the end-drought harvest.

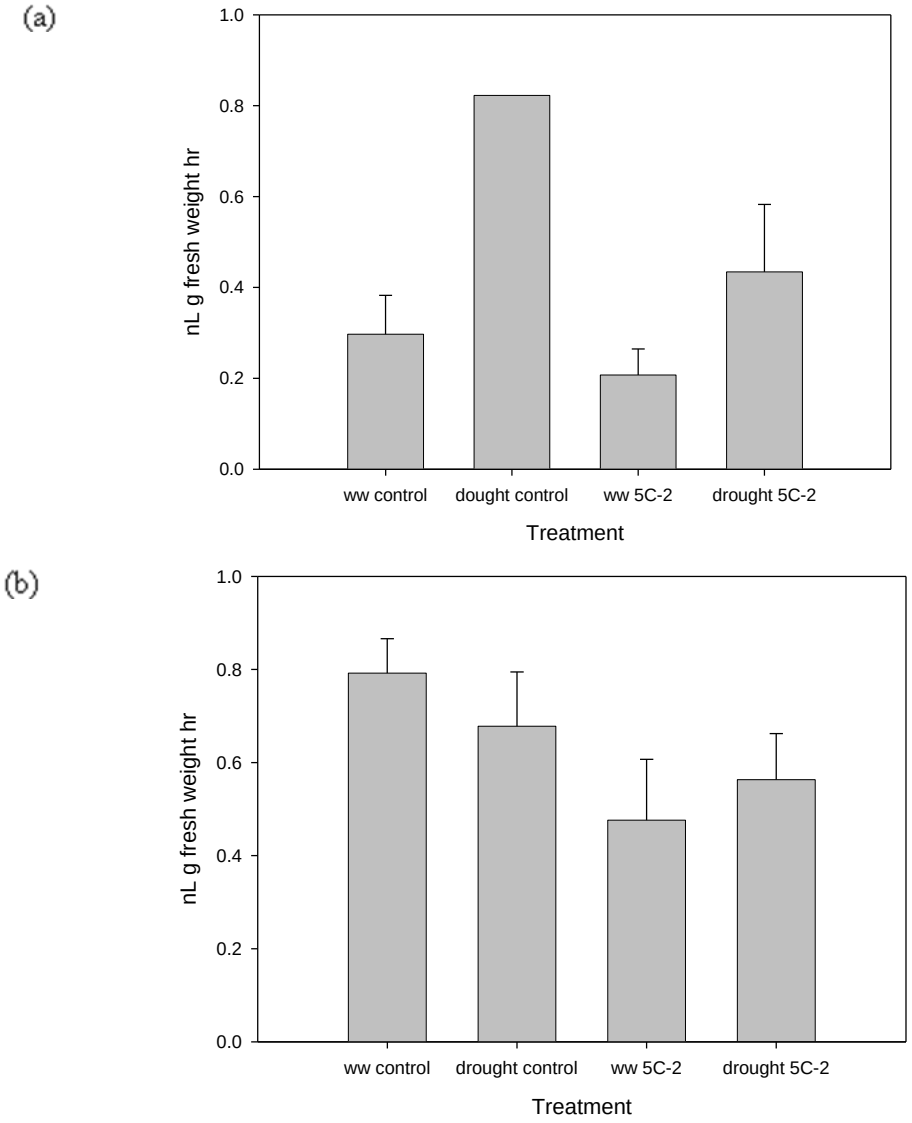


Figure 9. Foliar ethylene evolution at the end of drought (a) and final (b) harvests. No significant difference (p > 0.05) in treatments in leaf ethylene level at the end-drought harvest. (b) No significant difference (p > 0.05) in treatments in leaf ethylene levels at the final harvest.

ABA measurements were taken pre-drought, end-drought and at the final harvest. There was no significant difference ($p = 0.623$) between treatments pre-drought. Two-way ANOVA found both water treatment and bacterial inoculation with 5C-2 had significant effects ($p = 0.000$, $p = 0.035$ respectively) and a significant interactive effect ($p = 0.024$) on foliar ABA levels at the end-drought period (Fig. 9). Water treatment had a significant effect ($p = 0.037$) on foliar ABA level at the final harvest, foliar ABA was higher in the droughted plants, there was no effect of 5C-2 ($p > 0.05$).

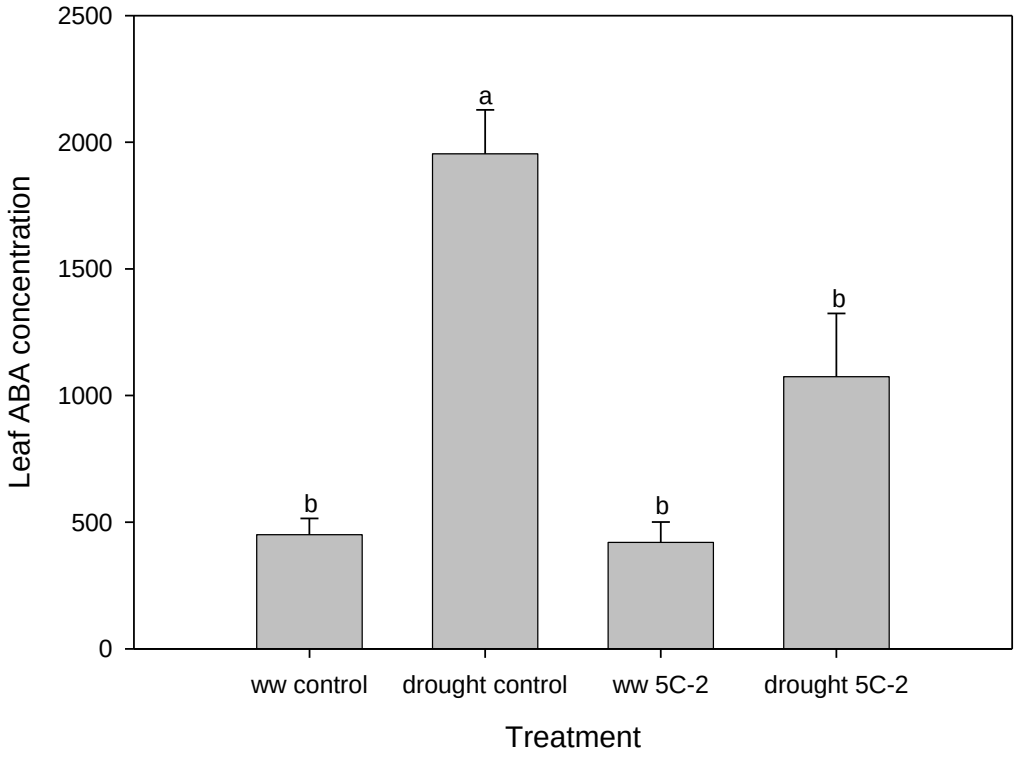


Figure 10. End-drought leaf ABA levels. Mean data are shown for each treatment ± error of mean. Letters denote significance between the treatments. Both water treatment and 5C-2 inoculation had significant effects ($p < 0.05$) on ABA levels at the end-drought period. Different letters (above the bars) indicate significant ($P < 0.05$) differences according to 1-way ANOVA

Lee Farm field trial

There was no significant difference ($p > 0.05$) in water treatment or bacterial treatment on SFW at the final harvest of the field trial at Lee Farm 117DAP (Fig. 10).

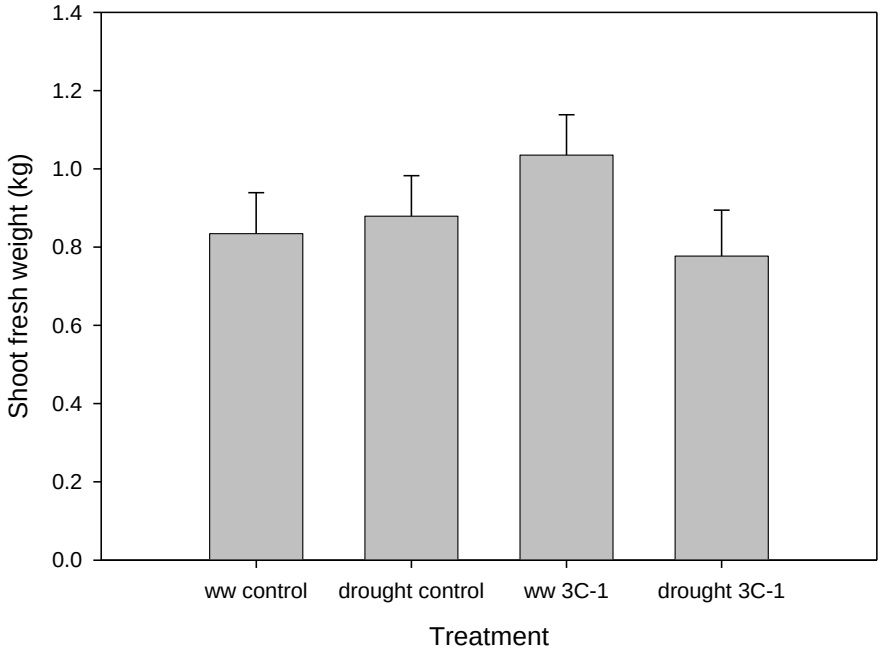


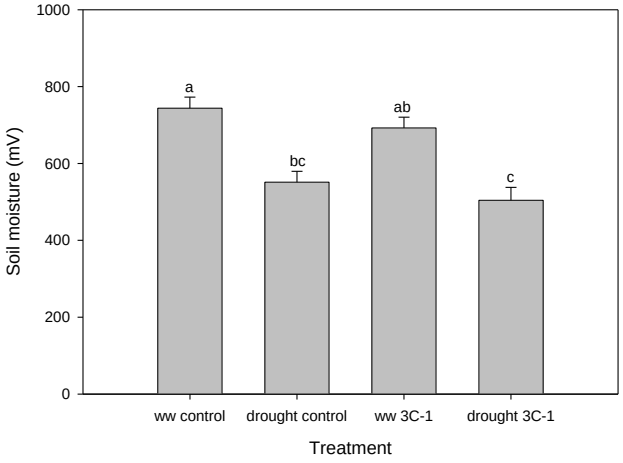
Figure 11. Shoot fresh weight of final harvest of field trial plants at Lee Farm. There was no significant difference ($p > 0.05$) in SFW between any of the treatments. Data are means ± SE of n = 17 control, n = 20 3C-1 replicates

Three days after watering ceased, the soil moisture of the droughted plots was significantly lower ($p < 0.001$) than the well watered plots (72DAP). There was no effect of 3C-1 upon soil moisture levels ($p > 0.05$) nor was there an interactive effect between the two variables 72DAP. Levels of soil moisture remained significantly lower ($p < 0.05$) in the droughted plants throughout the course of the experiment (Fig. 11a)

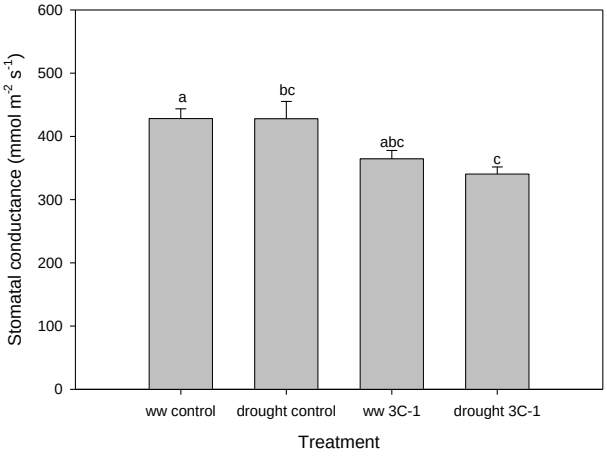
Rhizobacterial inoculation with 3C-1caused stomatal conductance to significantly decrease ($p < 0.000$) 72DAP, but there was no significant effect of water deficit ($p = 0.496$) (Fig. 11b). Conversely 99DAP, 3C-1 had no statistically significant effect on stomatal conductance ($p = 0.1$), whereas there were significantly lower ($p = 0.005$) levels of stomatal conductance in the droughted plants (Fig. 11c).

(a) ABA measurements were taken 99DAP but there was no significant difference ($p > 0.05$) in levels between the different treatments (data

(b)



(c)



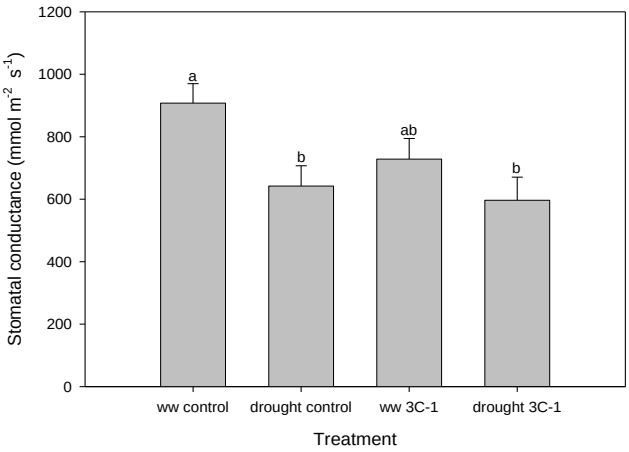


Figure 12. (a) Soil moisture was significantly lower ($p < 0.001$) in the droughted plots than the well watered plots irrespective of treatment with 3C-

1.(b) 3C-1 significantly reduced ($p < 0.001$) stomatal conductance in both well watered and droughted plants 72DAP, with no significant effect of water treatment ($p = 0.496$). (c) 3C-1 had no significant effect on stomatal conductance ($p = 0.1$) 99DAP, but it was significantly ($p = 0.005$) lowered in the droughted plants at this time.

Produce World field trial

There was no significant difference in head fresh weight between in the control and 3C-1 treated calabrese ($p = 0.155$) (Fig. 12). Those that had been treated with broth had significantly reduced head fresh weight compared to the control ($p = 0.007$), but not compared to the 3C-1 inoculated plants ($p = 0.397$).

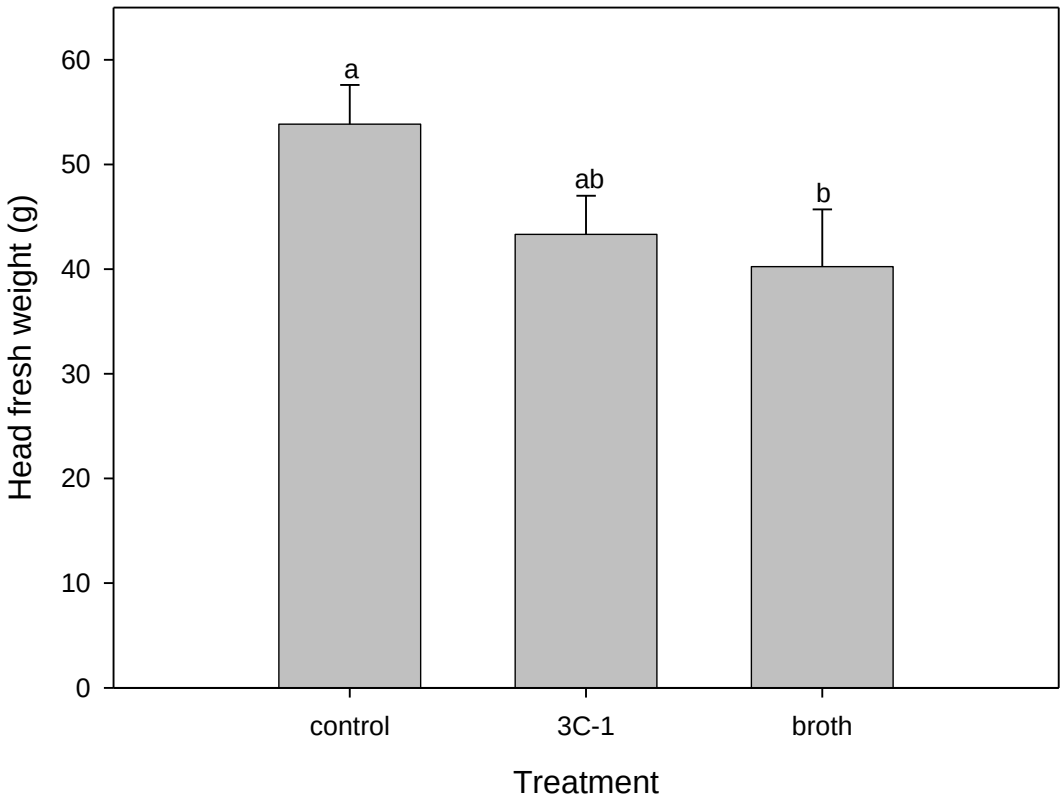


Figure 13. There was no significant effect ($p = 0.155$) of 3C-1 treatment on head fresh weight compared to the control. Broth treatment significantly decreased head fresh weight compared to the control ($p = 0.007$).

DISCUSSION

The ACCd producing PGPR *V. paradoxus* 5C-2 has been previously shown to promote growth in pea, maize, *Arabidopsis thaliana,* and preliminary work has shown accelerated tuber sprouting in potato (Belimov, et al., 2009a; Belimov, et al., 2009b; Dodd, et al., 2009; Chen, et al., 2013). Furthermore, 5C-2 influences ABA relations *in planta,* although there are species differences which account for the variations in ABA partitioning and signaling, especially under soil-drying conditions (Belimov, et al., 2009a; Jiang, et al., 2012; Dodd, et al., 2009). The effects of these ACCd producing bacteria and drought stress upon calabrese will be hereupon discussed.

Physiological response

V. paradoxus 5C-2 had growth-promoting effects on calabrese when the seedlings were soil inoculated at seven days old with a concentrated solution. However, growth inhibition of calabrese seedlings occurred when 5C-2 bacteria were added to the soil at the time of seeding. Contrastingly, when 3C-1 was added at seeding to calabrese at Fountain Plants, it increased SFW by 18%.

The difference in time of inoculation and growth-promoting effects has not been previously studied in the published literature, which indicates that the method of inoculation of the seeds/seedlings may be a factor in explaining why these results did not mirror previous research (Belimov, et al., 2009a) (Dodd, et al., 2009) (Jiang, et al., 2012) In the pot trial, a very concentrated inoculum was added directly to the soil surrounding the seed, whereas at Fountain Plants the concentrated bacterial solution was diluted to OD and watered onto the soil, and was thus less concentrated around the seed. Previous methods of application of these PGPR have been in solution (Belimov, et al., 2009a) (Jiang, et al., 2012) (Chen, et al., 2013).

As previously discussed, ACCd production by these PGPR manipulates the concentration of ACC within the root/seed exudates. Consequently the ACC levels within the root/seed decrease leading to a reduction in ethylene biosynthesis. Although the hormones ABA and gibberellins (GA) (Finch-Savage, 2006) are primarily responsible for seed germination, ethylene does play a role alongside GA in counteracting the inhibitory effects of ABA, although how it does so is not well understood (Linkies, et al., 2009; Linkies & Laubner-Metzger, 2011; Matilla, 2007). ACC has been found to decrease the ABA sensitivity of seeds, thus helping to promote germination (although it is by no means a key factor) (Ghassemian, et al., 2000; Linkies & Laubner-Metzger, 2011). Here it is proposed that these ACCd producing rhizobacteria

have an inhibitory effect on seed germination at high concentrations due to the reduction in endogenous seed ACC, thus ‘slowing down’ the early processes of seedling development, which is why opposite effects of bacterial inoculation at seeding compared to inoculation at seven days old were found .

Despite initial growth promotion of the calabrese seedlings treated at Fountain Plants (Table 1), by full maturation (after transplantation into the field under commercial conditions) there were no significant differences in head weight with rhizobacterial inoculation (Fig. 13). Seed yield of pea was increased with 5C-2 inoculation under soil drying, but there was no impact under well-watered conditions (Belimov, et al., 2009a), therefore if a difference in head weight were to be seen, it would be more likely under drought-stress. However, the ability of these bacteria to survive in the field is questionable because of the high levels of other microbes living in this environment competing for the same nutrients and sites on the root system of the plants (Lugtenberg & Kamilova, 2009).

Regardless of the measures taken to avoid cross-contamination of the bacteria from one plot to another, lateral transfer on the bodies of macrofauna, such as earthworms would have been likely (Doube, et al., 1994). Therefore it is possible that the ACCd producing bacteria spread to the control plants. However, again, due to high levels of competition in the rhizosphere microbiome, it is unlikely that thse rhizobacteria would have induced significant effects upon the plants (Lugtenberg & Kamilova, 2009). Therefore the rhizobacteria either did not survive post-transplanatation, or their growth-promoting effects only influence specific developmental stages of this crop. Nevertheless, despite no evidence of long-term effects, early growth-promotion would be beneficial to companies such as Fountain Plants, who supply large volumes of seedlings to commercial farms. Initial growth acceleration of these crop plants would enable a greater turn-over of stock.

Interestingly, broth treatment signficantly decreased head weight compared to the control plants. The bacteria were suspended in the same broth that was watered onto the seeds. The broth therefore does not appear to inhibit growth promotion in the early stages of development, but unfortunately seedling measurements of broth treatment were not taken because of a lack of space for transportation of the seedlings trays back to Lancaster. In this instance, the reduced head weight with broth treatment in the field is most likely to be explained through field-scale soil heterogeneity of soil moisture levels/nutrient availability, (plots 6,7 and 8 had few harvestable heads at the time of measuring, see Fig. 3 for the location of broth treated plants compared to control and 3C-1 inoculated of plots 1-5) influencing yield irrespective of treatment (Patzold, et al., 2008).

Half of the calabrese seedlings grown at Lee Farm were subject to drought stress. Drought and soil-drying reduces growth and yield of plants (Chaves,

et al., 2003; Lisar, et al., 2012), however incoulation of some plant species with the ACCd producing rhizobacteria 5C-2 minimises the stress response (Belimov, et al., 2009a; Belimov, et al., 2009b; Dodd, et al., 2009; Jiang, et al., 2012). Despite an imposed drought treatment to half of the plots under the polytunnel, with significant reductions in soil moisture (Fig. 12a), there was no significant effect on shoot fresh weight at the final harvest by either the drought stress or inoculation with 3C-1.

Despite the elimination of the majority of rainfall from the crops by the polytunnel, two sides were open, which meant some of the edge plants would have received some rainfall throughout the duration of their growth in the field. Also, as the surrounding soil became saturated, water subsequently percolated and flowed laterally through the soil to moisten the outer edges of the polytunnel (O'Green, 2012) . Concequently, the dry bed at the side of the tunnel would have experienced wetting. There were also a number of leaks in the irrigation system which meant some areas of the dry beds did not fully experience drought.

Developmental plasticity of root architecture would have enabled plants to overcome top-soil drought if water was available further down in the soil profile (distance limiting) (Frensch, 1997; Malamy, 2005; Bengough, et al., 2011). Under soil drying, root growth is usually less inhibited by shoot growth (Sharp, et al., 2004), and it has been demonstrated that phytohormonal control (primarily involving ABA) of longitudinal cell elongation at the root tip allows root extension, in spite of decreased cell turgor under water stress (Sharp, et al., 2004).These interaction are complex however as ABA is also required for inhibition of root growth under water stress (Sharp & LeNoble, 2002a). The water table at Lee Farm may have been relatively high (not measured) which would have enabled the plants to reach water despite top-soil drying.

Similarly to the harvest data at Lee Farm, there was no significant difference in SFW at the final harvest of the pot trial (Lancaster Uni). Drought stress at any stage of *Brassica oleracea* growth has been shown to reduce yield, but the most sensitive stages of development to drought stress are during head formation and enlargement and early growth (Singh & Alderfer, 1966). Maurer (1976) imposed five watering regimes to broccoli (Brassica oleracea) plants, (including the effects of drying and rewatering as with this pot experiment) and constrastingly found that the imposition of soil drying at difference stages in broccoli development did not reduce yield except during head formation, but did reduce shoot, root and leaf weight (Maurer, 1976). In field trials, Maurer (1976) also found that even if soil drying had been imposed earlier in development, if plants recevived adequate soil water during head formation there was no impact on yield compared to plants which were well watered throughout (Maurer, 1976).

The pot trial data complements the results of Maurer's (1976) research as shoot growth was limited during the drought period, but recovered by the final harvest, with no signficant effect on shoot fresh biomass or yield (head weight).

Drought treatment significantly decreased SFW, SDW and LA at the mid-drought and end-drought harvests of the pot trial compared to the control, but there was no significant effect of rhizobacteria. The physiological responses of the plants to drought treatment follows the typical pattern of drought-stress response in plants (Bray, 1997; Lisar, et al., 2012). For example, the number of leaves shed was greater in the droughted plants resulting in decreased leaf area (data not shown), and leaf wilting was visually evident due to a reduction in leaf water potential, which also limits growth and decreased stomatal conductance (Lisar, et al., 2012). However, the lack of significant effect of inoculation with 5C-2 is surprising as previous soil-drying research has shown growth-promoting effects of ACCd producing PGPR in droughted plants (Dey, et al., 2004; Belimov, et al., 2009a; Belimov, et al., 2009b; Dodd, et al., 2009) (Jiang, et al., 2012) due to the reduction of ACC in the roots, thus limiting the production of 'stress ethylene' (Glick, 2005).

A possible explanation for the lack of response of calabrese to 5C-2 is again as a result of the timing of application. Seedlings were incoulated 31DAP; much later than inoculation for the other trials, and thus if growth-promotion is only initiated in the initial stages of development, 5C-2 would have had no effect. However, this does not explain why there was no effect of drought stress response.

Drought stress response in calabrese may not be as dependent on ethylene synthesis as with the other species (pea, maize, potato) investigated so far. This is something which could be investigated further. It would be advantegous to analyse systemic ACC and ethylene levels throughout the plants life cycle and during drought stress. Difference have been shown in systemic ABA signalling in response to 5C-2 (Belimov, et al., 2009a; Dodd, et al., 2009), therefore similarly, there may also be species specific differences in ethylene signalling in response to treatment which will be further addressed later in the dicussion.

Hormonal response

Throughout the drought period of the pot trial, stomatal closure was significantly decreased as a result of drought treatment. Mid-drought, 5C-2 treatment also had a significant role in decreasing stomatal conductance independently of water treatment. Foliar ABA levels (taken from the newest most fully expanded leaf) were significantly higher in the droughted plants, which correlates with the increase in stomatal closure causing reduced levels

of stomatal conductance. However, 5C-2 also significantly decreased foliar ABA in the droughted plants compared to the control in the end-drought harvest of the pot trial. It is noted that there was no coincident increase in stomatal conductance at this point. Alongside this, leaf water potential and osmotic potential of calabrese in the pot trial were decreased under drought stress.

Similarly in the field trial at Lee farm, 3C-1 treatment caused a significant decrease in stomatal conductance in both well watered and droughted plants on one day of measurements, and on the other, drought treatment significantly increased stomatal closure.

In order to conserve water, nutrients and carbohydrates, plants respond to stresses such as drought through stomatal closure, which is mediated by ABA signalling in response to changes in the rhizospheric environment (Wilkinson & Davies, 2002). Stomatal conductance is directly correlated with stomatal opening, as it is the measure of CO_2 entering and water vapour exiting the stomata for gas exchange and the regulation of leaf temperature (Pospisilova, 2003).

ABA has a central role in stomatal closure in response to drought, which can be mediated from hydraulic or chemical signalling (Wilkinson & Davies, 2010). Roots in drying soil can synthesise and/or transport ABA in the xylem to the shoots triggering ABA-induced stomatal closure (Wilkinson & WJ, 2009). Ethylene is also an effector for stomatal closure (Pallas & Kays, 1982). However, ABA and ethylene cross-talk in the guard cells, whereby ethylene impairs the regulation of, and inhibits, ABA-induced stomatal closure (Tanaka, et al., 2005). It has been suggested that ethylene ensures there is some supply of carbon dioxide for photosynthesis by keeping the stomata half-opened under longer periods of drought stress to maintain some level of growth (Tanaka, et al., 2005).

Therefore, the decreases in stomatal conductance under drought conditions in the pot trial arose as a result of ABA chemical and hydraulic signalling leading to stomatal closure, confirmed by the high levels of foliar ABA in the droughted plants (Wilkinson & Davies, 2010), However, rhizobacterial treatment also significantly decreased stomatal conductance and foliar ABA in both well watered and droughted plants, and at the same time had near-significant effects on lowering foliar ethylene.

The interactive effect of ABA and ethylene levels due to ACCd producing rhizobacteria is interesting. Comparing Fig. 9a (ethylene end-drought) and Fig. 10 (ABA end-drought), the relative levels of ABA and ethylene between treatments follow identical patterns. As aforementioned, previous work has shown decreases in ABA with 5C-2 inoculation and the effects of ACCd on decreasing ethylene biosynthesis appear to correlate with the literature. However, to fully understand the effects of these phytohormones in response

to these ACCd producing rhizobacteria, a more in depth analysis of root to shoot concentrations of ACC, ethylene and ABA in the xylem and organ tissues is required. A model the flow of hormones around the plant would allow a greater comparison of this data with the literature (Belimov, et al., 2009a; Dodd, et al., 2009; Jiang, et al., 2012).

Another point to note is the difference in levels of ethylene between the end-drought and final harvest. At the final harvest levels were much higher. This is a typical response in broccoli/calabrese as deteriorates rapdily after flowering, with the most obvious feature of decline being chlorophyll degradation in which ethylene plays an important role. (Tian, et al., 1994) (Gapper, et al., 2005).

CONCLUSION

The interactions between ACC, ethylene and ABA and ACCd producing rhizobacteria are complex due to the cross-talk between the hormones, particularly because at different concentrations, stages of plant development, in different tissues, and under different environmental stresses these hormones vary greatly in their physiological responses and interactions with one another (Tanaka, et al., 2005; Wilkinson, et al., 2012). The effects of the ACCd producing rhizobacteria on calabrese varied between the trials conducted in this study, seemingly according to the timing of application. The hypothesised effects of 5C-2 and 3C-1 in reducing ethylene biosynthesis in response to drought stress do appear to occur, although more detailed measurements of xylem, phloem, root and shoot ACC concentrations, and ethylene / ABA signalling would be benefical. There appears to be distinct species-specific responses to drought stress since some of the data on ethylene and ABA concentrations (Fig 9, 10) were at odds with much of the published literature on physiological responses to ACCd –containg rhizobacteria (Belimov, et al., 2009a; Dodd, et al., 2009; Jiang, et al., 2012; Chen, et al., 2013). The growth-promoting effects of rhizobactieral inoculation on calabrese were limited to seedlings. Seedling growth promotion with treatment of *Variovorax paradoxus* 5C-2 or 3C-1 would be beneficial to seedling growers, such as Fountain Plants, as it would decrease the time span between seeding and selling to farmers.

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APPENDIX A

Figure 14. A picture showing the set up of seedling trials. Trays were rotated daily to minimize tray effects due to sunlight for example, as seen here.

APPENDIX B

Figure 15. A picture of the pot trial just after the seedlings had been transplanted 24DAP. The plants were in 4l pots with John Innes No. 2 substrate. The plants were inoculated with 5C-2 seven days later.

APPENDIX C

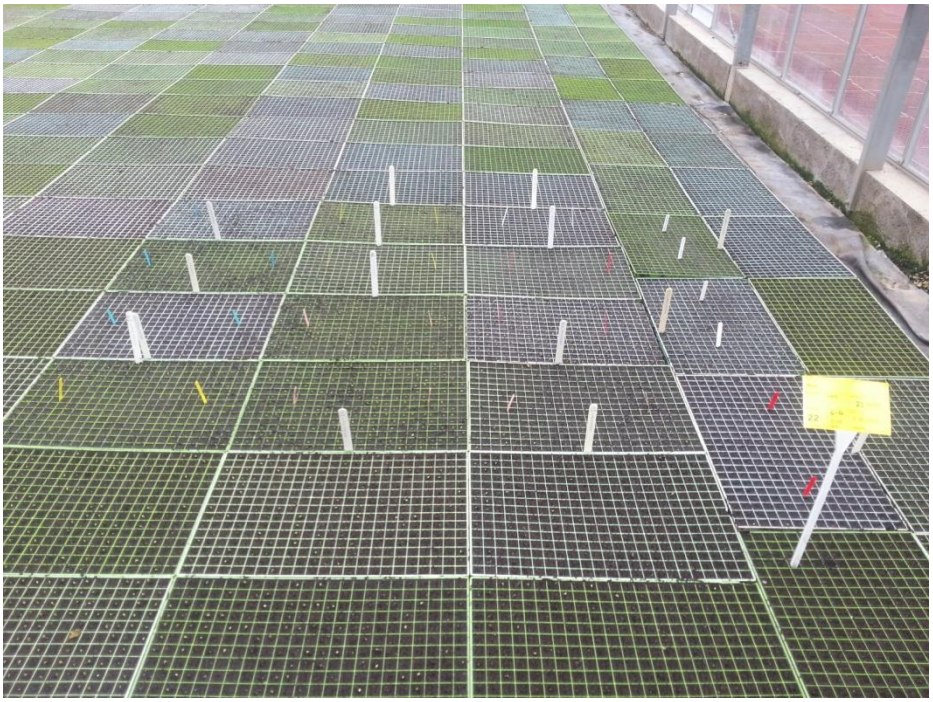

Figure 16. (a) 3C-1 inoculum was diluted to an OD 0.2 on site using tap water. (b)The diluted inoculum was poured evenly across each tray. (c) The experimental trays were places in the middle of other trays to eliminate edge effects within the glasshouse.

APPENDIX D

Figure 17. In this photo of plot 8, the ploughing marks are clearly visible across the field. The 48 day old seedlings were hand transplanted 50cm apart.

APPENDIX E

Figure 18. Seedlings have just been hand transplanted into the field site at Lee Farm (Lancs.). The polytunnel is clearly visible, used to exclude rainfall from the site. Irrigation piping is set up down the tunnel in four lines (three pipes per line), two alternate lines were turned off to implement drought stress.

APPENDIX F

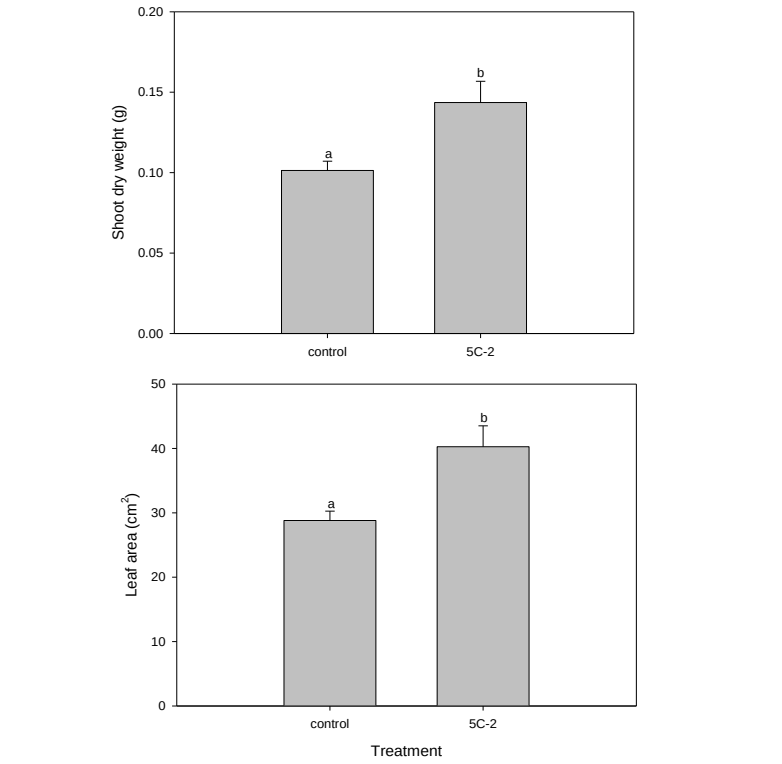

Figure 19. (a & b) show the mean SDW and LA values for a seedling trial. 5C-2 inoculated plants were treated 7DAP. (c) A photograph of the same seedling trial. The significant increase in above ground shoot biomass can be seen clearly in the treated tray on the left hand side with the yellow tab.