Rhizobacterial colonisation of, and physiological effects on, peanut (*Arachis hypogaea* L.) under water-limited conditions

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Submitted September 2013

This dissertation is submitted in partial fulfillment of the degree of MSc Sustainable Agriculture and Food Security

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ABSTRACT

Background and Aims: Increasing agricultural productivity under water-limited conditions may be achieved by optimising traits conferring high growth, combined with favourable soil management strategies. Increasing crop growth requires further understanding of physiological responses under defined water deficit scenarios. Applying plant growth promoting rhizobacteria (PGPR) has been proposed as an effective, sustainable method. The PGPR *Variovorax paradoxus* 5C-2 increased growth and yields of a temperate legume under water-limited conditions. This study aimed to: (1) determine whether *V. paradoxus* 5C-2 colonises peanut roots in a tropical soil; (2) identify genotypic differences in peanut responses to mid-season drought and re-irrigation; and (3) investigate whether *V. paradoxus* 5C-2 affects peanut growth in irrigated and drying soil.

Methods: A peanut crop was inoculated with *V. paradoxus* 5C-2 in a field experiment at Khon Kaen University, Northeast Thailand, with uninoculated controls. Mid-season drought (MSD) was imposed by withholding irrigation between 30 and 60 days after planting (DAP), whilst irrigated controls were maintained at field capacity (FC). The crop was monitored up to 90 DAP, when all subplots were at FC. Root colonisation and physiological responses to the irrigation and rhizobacterial treatments were assessed for four genotypes.

Key Results: (1) *V. paradoxus* 5C-2 colonised roots of each genotype and proliferated for at least 37 days from inoculation; (2) Genotypes differed in their responses of relative water content (RWC), leaf area (LA) and shoot dry weight (DW) to MSD. MSD increased root relative growth rates (RGRroot) for all genotypes, but did not affect nodulation. Re-irrigation increased relative leaf expansion rates (RLER) for each genotype; (3) *V. paradoxus* 5C-2: promoted stomatal conductance of three genotypes prior to MSD; affected RWC during soil drying according to genotype; and decreased daily LER during MSD but increased it following re-irrigation. At 90 DAP, neither MSD or rhizobacteria affected shoot or root biomass.

Conclusions: The reduction in shoot biomass caused by MSD (which was promoted by 5C-2) was negated by increased growth rates following irrigation. Understanding the different responses to drying and re-irrigated soil, and effects of rhizobacterial inoculation, might inform the optimisation of different traits under alternative drought scenarios.

Keywords: Mid-season drought, peanut, ACC-utilising PGPR, leaf water relations, relative expansion rates, growth.

Word count: 9158

INTRODUCTION

Water-limited agriculture: targets and approaches

Globally, agricultural productivity is frequently limited by a lack of freshwater. In regions dominated by dryland agriculture, water deficit is usually experienced during crop reproductive growth, when the water demand is highest (Blum, 2009). With uncertain irrigation supplies, developing strategies to increase crop growth with reduced water will be crucial for sustaining yields.

Drought resistance and yield are complex characteristics. Breeding programs aim to identify and select for traits conferring the maintenance of high growth under water-limited conditions (with less available soil water than required for maximal evapotranspiration) (Manavalan et al., 2009). Progress remains limited by an insufficient understanding of the sensitivities of traits and their interactions to different severities and timings of soil water deficit, as well as climatic, soil and management conditions (Tardieu, 2012). Physiological responses which conserve water may be beneficial or alternatively detrimental to crop growth and associated yield. Whilst high water use efficiency (WUE; ratio of biomass to water use) may be beneficial under terminal and severe water deficit, it has been associated with reduced growth and yields under conditions with water available at the end of the crop cycle (Tardieu, 2012). The effective use of water (termed EUW; maximising soil water uptake and the proportion contributing to stomatal transpiration) may be an alternative target for crop breeding, which may be negatively correlated with WUE and is driven by root growth and the ability to sense drying soil locally and respond systemically (Blum, 2009).

Appropriate strategies for overcoming the growth and yield reductions caused by water deficits that affect peanut (and other crop species) are needed, particularly techniques that are accessible for farmers in economically disadvantaged regions (Reddy et al., 2003). Through improved mechanistic understandings, management practices can be developed which promote beneficial traits for optimal growth under particular water-limited conditions (Wilkinson and Davies, 2012). The application of plant growth promoting rhizobacteria (PGPR) offers a promising approach to achieving these targets (Dey et al., 2004; Belimov et al., 2009).

Crop growth under water-limited conditions

Beneficial traits depend on the specific water-limited conditions

Physiological traits associated with drought resistance have been investigated by distinguishing between avoidance and tolerance mechanisms

(Turner, 2001; Manavalan et al., 2009). Drought avoidance is indicated by isohydric behaviour, whereby water loss is restricted by partial stomatal closure and leaf water status is maintained, whilst productivity may be reduced due to restricted CO_2 assimilation. Drought tolerance, in contrast, is indicated by anisohydric behavior which describes the ability of plants to maintain high stomatal conductance despite lower water potentials; therefore plants maintain higher productivity and growth (Subbarao et al., 1995). Traits associated with both isohydric and anisohydric behaviours have been described in peanut (Songsri et al., 2009; Rowland et al., 2012). However, the usefulness of this classification is limited. The conclusion that plants conferring the greatest resistance to soil water deficits are able to switch between mechanisms associated with avoidance and tolerance, according to soil moisture contents (Domec and Johnson, 2012), was misleading since the plant responses also moderate changes in soil water availability (Tardieu, 2012). Understanding the water deficit scenario is crucial to defining the target traits associated with optimal growth, for different species and genotypes (Tardieu, 2012; Wilkinson and Davies, 2012).

The variability in physiological responses to water-limited conditions makes the selection of target traits for crop improvement challenging. For example, more sensitive stomatal closure is a drought tolerance feature under severe water deficit scenarios; however, under mild water deficits this response may be detrimental if the carbon assimilation has a greater influence on biomass production than the water loss (Wilkinson and Davies, 2012; Puértolas et al. 2013). However, as well as reduced productivity the trait is associated with increased canopy temperature, so whilst limiting transpiration, water loss via leaf-to-air diffusion may be enhanced; an effect that is prominent in warm climates (Wilkinson and Davies, 2012). This water loss is particularly prominent in rainfed (compared to irrigated) systems of peanut production, under which it negated the water conservation by stomatal closure (Reddy et al., 2003). Therefore drought tolerance and the effectiveness of water use may be reduced under certain conditions (Blum, 2009).

Systemic responses to water deficits

The distinction between physiological responses to water-limited conditions, and true adaptive mechanisms for mediating the effects to prevent growth limitation, is unclear (Xiong et al., 2006; Tardieu, 2012; Puértolas et al. 2013). Sensing rhizosphere conditions according to local (root) traits is fundamental to determining the effects of water limitation, and to the ability to moderate transpiration and leaf water status.

In addition to hydraulic adjustment, regulatory hormones, such as abscisic acid (ABA) and ethylene, have a central role in mediating local and also systemic responses, via root-to-shoot signalling (Dodd, 2005). Processes

interact, as the effectiveness of ABA signalling to stomatal guard cells and expanding leaves is dependent on the water flux maintaining xylem sap flow, whilst ABA may also increase hydraulic conductivity (Dodd. 2005; Wilkinson and Davies, 2012). Therefore alternative possibilities exist for reduced or maintained transpiration and stomatal conductance, and productivity, depending on the specific interactions between regulatory processes. Physiological effects are not mechanisms *per se* but may result in positive or negative yield responses in different species and genotypes, depending on the extent and timing of the water deficit (Tardieu, 2012).

Relative leaf expansion rates and shoot growth indicate sensitivity to soil water deficits

Many mechanisms and responses coexist to contribute to drought tolerance (Xiong, 2006). As a measure of growth processes, leaf expansion rate (LER) is an integrating behaviour reflecting the determination of canopy growth and plant resource partitioning, light interception, soil water acquisition and transpiration, and phenological stages (Van Volkenburgh, 1999). Final leaf size was described as the cumulative result of plant metabolism (Mielewczik et al., 2013), and leaf expansion has been classified as the most sensitive developmental response to water deficit stress (Van Volkenburgh, 1999), particularly during the vegetative growth stage (Tardieu, 2012). As with other traits, decreased LER has been interpreted as an adaptive mechanism conferring drought avoidance, whilst also considered to be a response to changes in hydraulic flux and hormone balances, and other biochemical processes. A reduction in LER can have a greater affect than stomatal conductance on regulating transpiration, and therefore soil water depletion, under water-limited conditions (Sadras et al., 1993). It may occur before, or without, detection of changes in transpiration and leaf water status (Zhang & Davies 1990; Tardieu et al., 1999), and also before changes in ABA concentration, suggesting alternative causes of reduced expansion (Dodd, 2005).

As well as dependency on soil water availability, relative expansion rates are determined by cell turgor and cell wall extensibility, and also vary between genotypes under favourable conditions (Lockhart, 1965; Tardieu, et al., 1999; Bacon, 1999). Soybean leaf growth was also likely to have been affected by leaf developmental stage, although the extent of this effect has not been reported (Mielewczik et al., 2013). Interpreting differences in leaf expansion can provide a valuable insight into the effects of multiple processes on growth under water-limited conditions, but is insufficiently understood for field-cultivated legume crops (Mielewczik et al., 2013).

Soil water content affects root traits

Mediation of root growth is a local response to water-limited conditions. Consistent with Blum (2009), maintenance of high root biomass under water-limited conditions has been identified as a key physiological trait contributing to high growth and yield of legume crops; the increased soil water acquisition was associated with more effective water use (Pimratch et al., 2008; Songsri et al., 2009; Puangbut et al., 2011). Establishing a deep taproot early in the crop season has been associated with improved growth in water-limited legumes (Manavalan et al., 2009). Inhibition of lateral root growth in response to regulation by ABA in drying soil has been argued to be a true adaptive response (Xiong et al., 2006).

In legume species specifically, the symbiotic association between plant roots and rhizobia, which can provide a considerable proportion of nitrogen (N) requirements via biological fixation of atmospheric N_2, is particularly sensitive to soil drying. For example, water deficit decreased N accumulation, biomass production and associated yield in peanut (Pimratch et al., 2008). Leaf turgor pressure may also affect phloem flow into nodules; therefore the sensitivity of nodulation formation to soil drying and adjusted hydraulic gradients is a systemic as well as local effect (Serraj et al., 1999).

Mid-season drought limits growth and yields

Mid-season drought (MSD) is particularly inhibitory to growth for a diversity of crops, since the period incorporates reproductive as well as vegetative growth; for example flowering (R1), pegging (R2) and early subterranean pod formation (R3) in peanut. An alternative scenario of pre-flowering drought has been shown to have no effect on pod yield (Puangbut et al., 2009; Jongrungklang et al., 2011), whereas the greatest reduction in yield has been associated with water deficit between pegging and early seed-filling (R5) (Nageswara Rao et al., 1985; Nautiyal et al., 1999). Inhibition of peg penetration due to dry soil directly limited pod development (Reddy et al., 2003). Root growth of peanut plants was significantly reduced by water deficits imposed between 20 and 50 days after planting (DAP) (incorporating early reproductive development), but not during other periods (Meisner, 1991), illustrating the relevance of early MSD to growth. Due to the timing at which meteorological drought occurs in relation to crop phenology, MSD frequently limits biomass production and yields of many crop systems (Blum, 2009), including peanut (Jongrungklangrung et al., 2012). Mid-season drought under experimental conditions therefore represents a realistic scenario of increasing water deficit over time, as evapotranspirational water losses are not replaced. With the greatest differences in responses to water deficit expressed during this period (Jongrungklang et al., 2012), MSD is of

particular interest for research and to breeders in order to identify traits conferring optimal growth to sustain yields.

Recovery of growth and yields with re-irrigation is uncertain

Depending on the drought scenario, stomatal conductance and LER may recover following re-irrigation (Sadras et al., 1993), although enduring reductions in absolute rates of expansion can also result from temporary water deficits (Tardieu et al., 1999). Peanut growth recovered from severe water deficit stress with leaf relative water contents (RWC) as low as 30 % (Babu and Rao, 1983). In contrast, the root-to-shoot ratio increased whilst nodule number and dry weight, and shoot N content, were significantly reduced in pot-cultivated peanut exposed to drying soil, and did not recover to the levels of the irrigated controls following re-irrigation (Furlan et al., 2012). Further investigation of multiple traits for different peanut genotypes, under the specific scenario of MSD followed by re-irrigation, could therefore increase understanding of plant water status and growth responses, and the traits conferring maintained or recovered productivity.

Increasing water-limited crop growth with plant growth promoting rhizobacteria (PGPR)

Opportunities to promote the traits for optimal crop productivity with novel soil management practices may enhance the achievements of genetic approaches (Davies et al., 2011). The application of plant growth promoting rhizobacteria (PGPR) has been advocated as a relatively low-cost, low-technology and environmentally safe management strategy that can enhance productivity in a diversity of crop species. In addition, it may ameliorate growth inhibition caused by biotic and abiotic stresses via a range of mechanisms (e.g. for review see Seharan and Nehra, 2011).

ACC deaminase- containing PGPR

Colonisation of root surfaces with PGPR can alter the plant hormone status, and specific interactions can be exploited to promote crop growth. Plant growth has been promoted by inoculation with PGPR containing the enzyme ACC deaminase, which hydrolyses 1-aminocyclopropane-1-carboxylic acid (ACC; the immediate biochemical precursor to ethylene) to ammonia and α-Ketobutyrate which are utilised by the PGPR as sources of nitrogen (N) and carbon (C), respectively. With lower concentrations of ACC, ethylene synthesis and concentrations in roots (local) and shoots (via systemic root-to-

shoot signalling) in response to soil drying are reduced, resulting in growth promotion (e.g. Dey et al., 2004; Belimov et al., 2009).

Plant responses to abiotic stresses such as water deficit depend on hormone balances (Wilkinson and Davies, 2012). For example, ABA can antagonize ethylene production and result in growth promotion (Sharp, 2002; Dodd, 2005). Alternatively, in a citrus species exposed to soil drying and subsequent rehydration, corresponding root and xylem concentrations of ACC and ABA indicated that increased ethylene production in response to drying soil was upregulated by ABA. This indicated a growth-inhibition effect of ABA in addition to the stimulation of partial stomatal closure (Dodd 2005) and restriction of lateral root development (Xiong et al., 2006). Since this interaction occurred at the level of ACC synthesis, reducing concentrations of ACC can inhibit ethylene evolution, and the resulting local and systemic responses which inhibit growth (Dodd 2005). Such studies of the fundamental controls on physiological responses to stresses, including water deficit, have identified the opportunity for manipulation using PGPR which utilise ACC deaminase (ACCd).

Plant growth promotion with Variovorax paradoxus 5C-2

The ACCd-containing PGPR *Variovorax paradoxus* 5C-2, isolated from the rhizosphere of Indian mustard (*Brassica juncea* L. Czern.), was selected for its high colonisation and competitiveness (Belimov et al., 2005). It promoted growth of diverse crop species under a range of experimental conditions and environmental stresses, including water deficit (Belimov et al., 2009; Sharp et al., 2011; Jiang et al., 2012). The mechanism for growth promotion was ACCd activity: pea (*Pisum sativum* L.) root and shoot biomass were significantly increased by inoculation with wild-type 5C-2 under well-watered and water-limited conditions, whereas an ACCd deficient mutant of 5C-2 had no effect. In drying soil, xylem ABA increased significantly in plants inoculated with wild-type 5C-2, compared to controls and plants inoculated with the mutant strain. This may have been an indirect effect of the increased shoot growth and associated transpiration, driving increased soil drying (Belimov et al., 2009). In contrast, Jiang et al. (2012) found that inoculation with (wild-type) 5C-2 reduced ABA concentrations in pea xylem and shoots, and significantly in roots. Potentially, inoculation could counteract the water-conserving adaptations of plants that are induced by increased local and systemic ABA concentrations under water-limited conditions (Jiang et al., 2012). To optimise opportunities for its application in agriculture, further study of the effects of 5C-2 on physiological and growth responses in different species and genotypes, under a wider range of environmental conditions, is necessary.

Opportunities for peanut growth promotion with V. paradoxus 5C-2

Specific benefits of ACCd-containing PGPR have been reported for legume species, as the symbiotic relationship with rhizobia is particularly sensitive to the ethylene response to soil drying (Saleemet al., 2007). ACCd-containing PGPR strains promoted nodulation in pot-cultivated mung bean, compared to uninoculated control plants (Shaharoona et al. 2006). With water deficit imposed on pea plants at flowering and pod development, the most sensitive periods, inoculation with strains of *Pseudomonas* containing ACCd reduced the drought-induced inhibition of shoot growth and seed yield that was observed in the uninoculated controls, purportedly due to the reduction in ethylene (Arshad et al., 2008); nodulation was not discussed.

From a peanut rhizosphere, Dey et al. (2004) isolated nine strains of PGPR of *Pseudomonas* spp. on the basis of ACCd activity. Compared to controls, inoculated peanut plants had higher nodulation, shoot N content, growth and yields. The plants were cultivated under full irrigation and the effects on peanut under water-limited conditions were not assessed. It was concluded that mutational analyses would be necessary to further characterise the mechanisms for growth promotion of the different strains (Dey et al., 2004); this approach contributed to understanding the importance of ACCd activity in *V. paradoxus* 5C-2 for legume growth promotion (Belimov et al., 2009). With a reduction of root ethylene in pea inoculated with 5C-2, the nodulation inhibition induced by water deficit was overcome, with systemic benefits such as increased seed N content (Belimov et al., 2009; Jiang et al., 2012). Of four plant species investigated (representing four families), 5C-2 colonisation was highest for pea (R. Teijeiro, unpublished data), suggesting possibilities for beneficial associations in other legume species.

Therefore, this study had three objectives:

1. **Establish whether the PGPR strain *Variovorax paradoxus* 5C-2 can proliferate in a tropical field soil and colonise peanut roots.**

Given that 5C-2 grew at 32 °C and was motile up to 36 °C *in vitro* (R. Teijeiro, unpublished data), was known to survive temperatures of *c.* 50 °C (A. Belimov, pers. comm.), and had particular benefits for a legume species amongst temperate crops, further research was warranted to advance knowledge of the potential application of 5C-2 in a tropical agroecosystem.

2. **Interpret genotypic variations in peanut responses to mid-season drought and re-irrigation, to identify which physiological traits contribute to optimal growth.**

The need for an increased understanding of how root and shoot traits and their interactions relate to improved growth under water-limited conditions in different peanut genotypes, to advance breeding for drought resistance (Pimratch et al., 2008), persisted. A knowledge gap remained for particular genotypes and responses to the specific MSD scenario. Whilst yield components have tended to attract most attention, an opportunity to consider in more detail the responses of leaf water status and expansion to MSD and re-irrigation was identified.

3. **Determine whether *V. paradoxus* 5C-2 promotes growth of different peanut genotypes under field conditions with full irrigation and mid-season drought.**

Research into the physiological effects of ACC-utilising PGPR on peanut was scarce, other than Dey et al. (2004) who did not investigate water-limited conditions. Physiological responses to 5C-2 had never been investigated for peanut, or for a crop cultivated within a tropical field soil.

MATERIALS AND METHODS

Experimental conditions, design and treatments

A peanut (*Arachis hypogae* L.) crop was grown at the Field Crop Research Station of Khon Kaen University in Khon Kaen province, Thailand (16°28'N, 102°48'E, 200 m above sea level) from April to August 2013. This period comprised the late dry season (at the time of planting) and the rainy season. The soil type is Yasothon series (Yt: fine-loamy; siliceous, isohypothermic, Oxic Paleustults). Soil temperatures at 0, 5 and 10 cm depths below the surface was measured frequently at different times of day before two of the treatments were imposed, and at 5 cm depth within 20 subplots from 14:30 hr at 30, 47 and 74 DAP.

The experiment had a 2 x 4 x 2 factorial randomised complete block design with four replications. Two irrigation regimes comprised the main-plot treatment (34.0 m x 27.5 m) with four genotypes and two rhizobacterial (5C-2) levels both as subplot treatments (5.0 m x 3.8 m). Plants were spaced at 50 cm between rows and 20 cm within rows.

Irrigation

Irrigation was supplied by a sub-surface drip irrigation system. Subplots were maintained at field capacity (FC) throughout the experiment for the FC treatment. Early mid-season drought (MSD) was imposed by withholding irrigation between 30 and 60 days after planting (DAP), with FC maintained during other periods. Rain-out shelters were used at night and during rainfall to control water availability during MSD. The volume of irrigation water required to replace the water lost via evapotranspiration (ET_{crop}) was calculated as described by Doorenbos and Pruitt (1992):

$$ET_{crop} = ET_o \times K_c$$

where ET_o indicates evapotranspiration of a reference plant under particular conditions and was calculated based on pan evaporation. K_c is the crop coefficient for peanut water requirement, which for this experiment ranged from 0.40.to 0.95 as plants developed, and was adjusted at the same time for all genotypes since they were selected for similar phenology. Surface (soil) evaporation (E_s) (mm) was calculated as:

$$E_s = \beta \times (E_o / t)$$

where β is a light transmission coefficient which depends on canopy cover; E_o (mm day^{-1}) is evaporation from a class A pan; and t is the time (in days) since the previous irrigation (Doorenbos and Pruitt, 1992). Irrigation was supplied every two or three days, in accordance with precipitation and

other crop management activities (details provided in Appendix 1). Measurements of soil water content were made every seven days, to enable regular monitoring, at 30, 60 and 90 cm depths via an aluminium access tube in each subplot, using a neutron moisture meter (Type I.H. II SER. N◦NO 152, Ambe Diccot Instruments CO. Ltd., England).

Genotypes

Four genotypes with contrasting responses to water deficit, but similarly short durations, were planted. KS 2 (KS), KKU 60 (KKU) and ICGV 98305 (ICGV) have low, moderate and high drought tolerance, respectively (Jongrungklang et al., 2012). A non-nodulating variety (NN) was also used, to assess effects independent of biological nitrogen fixation.

Bacterial culture and inoculation

The rhizobacterium *Variovorax paradoxus* 5C-2 was acquired from the ARRIAM Collection (St. Petersburg, Russian Federation; Belimov et al., 2005). Two rhizobacterial treatments were imposed: uninoculated controls (UC) and inoculation with *V. paradoxus* 5C-2 (5C-2). Inoculum was prepared by precultivation of bacteria on solid tryptic soy agar (TSA) for 3 days at 30°C, suspension in sterile tryptic soy broth (TSB) and incubation for 32 hours at 28 °C and 180 rpm^{-1}. A growth curve of absorbance (at 600 nm) against time informed the optimal time of incubation to ensure bacterial growth was still in the exponential phase (Appendix 2). To confirm that 5C-2 was applied at the target concentration of 10^{-7} to 10^{-8} cells ml^{-1} (e.g. Jiang et al., 2012), a sample of the inoculum was serially diluted (10-fold) to 10^{-8} and 50 µl aliquots were plated onto TSA, incubated at 30 °C and observed after four days.

Inoculations were carried out at 19 and 29 DAP between 16:40 and 20:30, when soil temperature was decreasing. On both occasions the last irrigation to FC had been approximately 28 hours before inoculation, to avoid the highest temperatures and the risk of slow infiltration and surface ponding of inoculum, if irrigation had been sooner or later, respectively. The 5C-2 was applied with diluted TSB to support movement towards, and colonisation of, roots. Inoculum was supplied at the base of 5C-2 treated plants, with volumes of 4.8 ml using test tubes at 19 DAP, and 3.2 ml using automatic pipettes at 29 DAP. These volumes of TSB, which had been diluted to the average concentration of TSB supplied with 5C-2, were applied to uninoculated controls (UC) using automatic pipettes.

Soil properties

To determine mean pH, soil samples were taken from 15 to 30 cm depth from one randomly selected subplot per replication. Sub-samples of 10 g of soil were suspended in 10 ml of distilled water and the pH was determined using an electrode and standardised pH meter.

Soil temperature was measured using a digital thermometer at the soil surface and 2, 5 and 10 cm depths, at two locations per sampled subplot. Before rhizobacterial inoculation, measurements were made between 07:00 and 18:00 on numerous days, representing different conditions, to establish the temperature range and a suitable time for inoculation. Soil temperatures of the subplots from which root samples were collected to assay colonisation (n = 20) were measured before, during and after MSD (at 30, 47 and 74 DAP).

Physiological, growth and root colonisation measurements

Time to germination and flowering, and extent of pegging at 45 and 60 DAP, were monitored to record any treatment differences in phenology (Appendix 1).

Leaf relative water content (RWC) was measured at 30, 45, 60, 61, 63, 74 and 90 DAP. The second leaflets of the second fully expanded leaves were taken from five plants per subplot and immediately sealed in plastic bags and kept in ice boxes. Fresh weights were determined, after which the leaves were floated in distilled water in petri dishes and maintained in dark conditions at 25°C for eight hours. Turgid weights were recorded and the samples were oven-dried at 80°C for 48 hours (when constant weights had been reached). RWC (%) was calculated as:

$$\text{RWC (\%)} = ([\text{Fresh weight} - \text{Dry weight}) / (\text{Saturated weight} - \text{Dry weight})] \times 100$$

Measurements of stomatal conductance were made as close to 30, 45, 60 and 90 DAP as meteorological conditions would allow. The second leaflets of the second fully expanded leaves (hereafter referred to as leaves) of two or three plants per subplot were measured (SC-1 leaf porometer, Decagon Devices).

Leaf growth was measured daily between 37 and 67 DAP, to assess the effects of mid-season drought and re-irrigation on leaf expansion rate (LER). Measurements of the second leaflet of expanding leaves were made for two plants per subplot, which were the nearest non-adjacent plants to the neutron

probe access tube, using a plastic ruler and avoiding leaf damage. Leaflet lamina growth rates were expressed as mm day^{-1}.

Five plants per subplot were harvested at 30, 60 and 90 DAP. Leaves were separated from stems for measurement of fresh weights (FW) and individual plant leaf area (LA) was calculated from the measurement of a sub-sample of approximately 10 % of total leaf FW **(**LI-3100C Portable Leaf Area Meter). Stems (with pegs at 60 and 90 DAP, whilst pods were removed) and leaves were separately oven dried at 80 °C for 48 hours, after which dry weights (DW) were constant. Nodules were removed from the roots of the same five plants harvested for shoot growth. Nodules and roots were separately oven dried at 80 °C for 48 hours to determine specific root nodulation (nodule dry weight per root dry weight). Roots were extracted using a monolith (50 cm x 20 cm x 50 cm) at 30, 60 and 90 DAP for one plant per subplot, carefully washed and oven dried at 80 °C for 48 hours to obtain dry weights.

Rhizobacterial colonisation was assessed 23 and 24, 33, 59 and 66 DAP. Root samples were taken from adjacent border plants within the same subplots for each assay, which represented each irrigation and genotype combination, and each replication, equally (n = 16). Root samples were also taken from UC subplots, representing each genotype, both irrigation treatments and each replication, to confirm the absence of 5C-2 (n = 4). Crown root sections were taken, since 5C-2 colonisation was highest on pea roots with greater distance from the root tip (R. Teijeiro, unpublished data). Root sections were thoroughly shaken to remove adhering soil particles and 0.5 g samples, comprising primary and secondary roots, were placed in sterile petri dishes. The samples were each homogenised with 1.8 ml sterile distilled water using a sterile mortar and pestle. Aliquots of 50 µl of ten-fold serial dilutions (to 10^{-6}, each with two replicates) were plated onto TSA, which had been supplemented with two antibiotics to which 5C-2 is resistant, kanamycin sulfate (30 µl ml^{-1}) and rifampicin (20 µl ml^{-1}), and also nystatin (40 µl ml^{-1}) to reduce fungal growth. Plates were incubated at 30 °C and 5C-2 colonies, identified according to morphological characteristics, were counted daily for up to four days and expressed as colony forming units (CFU) per gram of root fresh weight (FW).

Data analysis

All data analysis was performed using SPSS 20. Students' unpaired t-test was used to assess treatment differences in soil temperature. Regression analyses were performed on log-transformed data for determination of relative expansion rates. ANCOVA, and ANOVA with Bonferroni post-hoc tests (which accounted for multiple testing) determined the significance of differences in physiological and growth parameters between the three experimental factors, and the specific significant interactions. Highest adjusted R^2 was the best-fit criterion for the ANOVA models and $P \leq 0.05$ indicated significant differences.

RESULTS

V. paradoxus 5C-2 colonised peanut roots under tropical field conditions

Soil pH and temperature

At planting, within the upper 30 cm the soil had pH 6.84 (mean, with standard error of 0.06). This was within the range that supported optimum growth and motility for 5C-2 *in vitro* (R. Teijeiro, unpublished data).

Minimum soil temperatures at 5 cm depth exceeded 28°C, which was previously identified as optimal for 5C-2 growth, and exceeded 40°C under MSD at which point 5C-2 growth ceased *in vitro* (R. Teijeiro, unpublished data). The greatest difference in soil temperature between FC and MSD subplots was recorded at 47 DAP, approximately the middle of the MSD period, when the mean temperature in the MSD subplots was significantly ($P < 0.001$) higher (by more than 10 °C) than subplots maintained at FC (Table 1).

Table 1. Soil temperature (°C) before (30 DAP), during (47 DAP) and after (74 DAP) the mid-season drought treatment. Measurements were made at 5 cm depth for all subplots from which colonisation of 5C-2 was assayed, with one UC per replication; data are means of two locations per sampled subplot ($n = 40$). Measurements were made from 14:30 on each occasion and under similar conditions (sunshine, and without irrigation or precipitation within the previous *c.* 24 hours).

	30 DAP		47 DAP		74 DAP	
	FC	**MSD**	**FC**	**MSD**	**FC**	**MSD**
Minimum	35.6	36.9	35.0	42.6	35.2	34.5
Maximum	37.5	43.1	38.8	50.0	39.9	40.7
Mean	36.5	40.5	37.0	47.6	38.1	37.4
SE	0.13	0.36	0.24	0.50	0.31	0.40

The temperatures recorded at different depths in the soil profile, and at different times of day, informed the volume of inoculum and the timing of inoculation likely to optimise 5C-2 root colonisation and proliferation (Figure

1). Whilst higher inoculum volumes would have reduced local soil temperatures, observations of low rates and shallow depths of infiltration in soil near to FC, and also in very dry, hydrophobic soil (using a colour dye), indicated that limiting inoculum volumes was desirable.

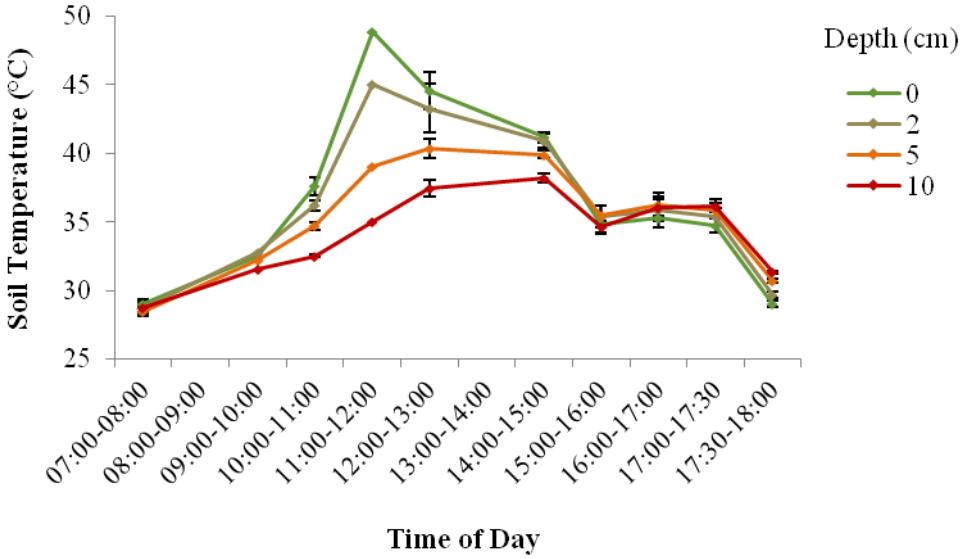

Figure 1. Soil temperatures at different times of day, at four depths below the soil surface. Data are means ± SE of measurements made on different days until re-inoculation, representing a range of atmospheric temperatures (27.7 to 37.8 °C) and soil water contents. Absence of error bars are for n = 1; otherwise n = 6 (12:00-13:00, 14:00-15:00), 8 (10:00-11:00, 17:00-17:30, 17:30-18:00), 10 (07:00-08:00, 15:00-16:00) and 16 (16:00-17:00). The separate data points between 17:00-18:00, and the increased n later in the day, were necessary to identify an appropriate time for the rhizobacterial inoculation (*c.* 28 °C at 2 cm depth).

Variovorax paradoxus 5C-2 successfully colonised peanut roots

In all four assays, *V. paradoxus* 5C-2 colonisation of peanut roots was detected. This indicated its persistence in the rhizosphere for at least up to 37 days after inoculation, including proliferation throughout the MSD period during which particularly high soil temperatures were observed (Table 1).

Colonisation assayed four days after the first inoculation was considered to be too low to potentially have growth-promoting effects. Better colonisation was detected four days after re-inoculation since 5C-2 was detected in twice as many of the sampled subplots, and for all genotypes (Table 2).

Table 2. Summary statistics for log colony forming units (CFU) g^{-1} root FW and detection of 5C-2, by assay, with genotype and irrigation treatments combined (*n* = 16). 5C-2 was not detected on roots of the UC plants assayed (*n* = 4).

	1 (23/24 DAP)	**2 (33 DAP)**	**3 (59 DAP)**	**4 (66 DAP)**
Mean	1.04	2.45	2.17	1.29
SE	0.41	0.55	0.42	0.45
Min.	0.00	0.00	0.00	0.00
Max.	4.00	6.00	5.00	5.00
n subplots (of 16)	5 (31%)	10 (63%)	11 (69%)	6 (38%)
Genotypes (of 4)	3	4	4	4
Replications (of 4)	4	4	4	4

Populations of 5C-2 ranged from 10^{-2} to 10^{-5} CFU g^{-1} root FW. The concentration of 5C-2 detected on peanut roots four days after re-inoculation was at least three orders of magnitude lower than the concentration of the inoculum supplied. Incubating inoculum aliquots on TSA confirmed growth of 5C-2 at a dilution of 10^{-8} after four days. Thus, the lower concentrations were a consequence of cell death following (and perhaps during) application to the field soil. However, since 5C-2 was not detected in all subplots, this reduced the mean CFU g^{-1} root FW of the genotype treatment groups. Recalculating mean colonisation by including only the plants with confirmed 5C-2 presence indicated that concentrations were as high as 10^{-6} CFU g^{-1} root FW, observed four days after re-inoculation (Assay 2) (Figure 2).

Based on all subplots sampled, populations tended to decrease with time between Assays 2, 3 and 4. In several cases 5C-2 was recovered from subplots which had no colonisation in the previous assay, indicating variability between plants within as well as between subplots. Similarly, an absence of 5C-2 for Assay 2 in subplots with presence recorded from Assay 1 suggested that the improved colonisation following re-inoculation was not simply a cumulative effect. Based on the confirmed root colonisation, the

population increased during MSD for KS only, and was at least one order of magnitude higher compared to other genotypes (Figure 2).

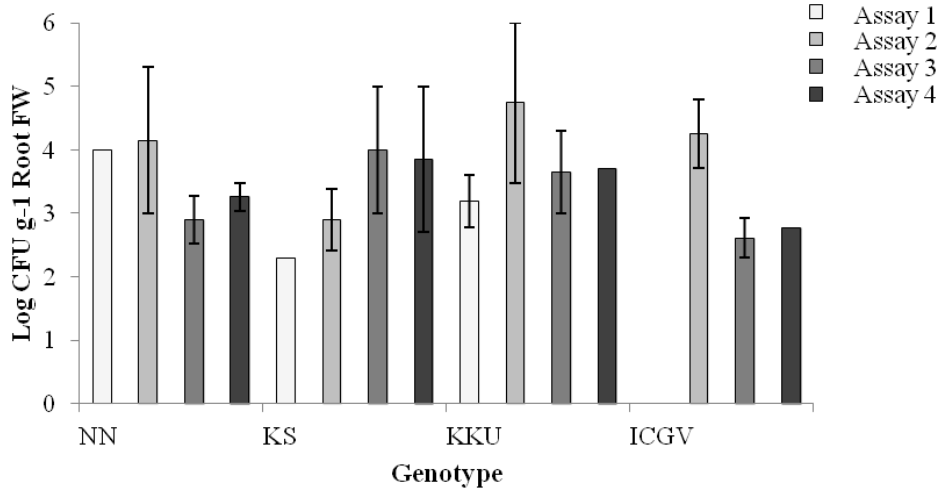

Figure 2. Populations of 5C-2 assayed on four occasions. Data are means ± SE of the samples for which 5C-2 colonisation of the roots was confirmed, with data for irrigation treatments combined. For assays 1, 2, 3 and 4 respectively, *n*: NN: 2, 1, 4, 2; KS: 1, 3, 2, 2; KKU: 2, 3, 2, 1; ICGV: 0, 3, 3, 1. Between assays 2 and 4 the mean population increased by 33 % for KS, whereas decreased for other genotypes, by 26 % on average (± SE of 4.4). The differences between assays were not significant (*P* = 0.277).

In all assays, variability within treatment groups was high and there was no difference in colonisation of the four peanut genotypes. Colonisation assayed for plants exposed to MSD was lower from Assay 4 (which followed re-irrigation) compared to Assay 3, which was consistent with the reduction in 5C-2 concentrations with time, observed across irrigation treatments. The 5C-2 was more consistently detected on roots of plants from MSD compared to FC subplots, in both assays. Furthermore, the highest concentrations were detected from plants that had been exposed to MSD (of 10^{-5} cells g^{-1} root FW, for KS). Therefore the MSD conditions supported 5C-2 proliferation, despite higher rhizosphere temperatures.

Physiological responses to mid-season drought (MSD), and effects of 5C-2

Soil moisture content

Volumetric soil moisture content was reasonably consistent throughout the experiment, except for the reduction between 30-60 DAP in the MSD treatment (Figure 3).

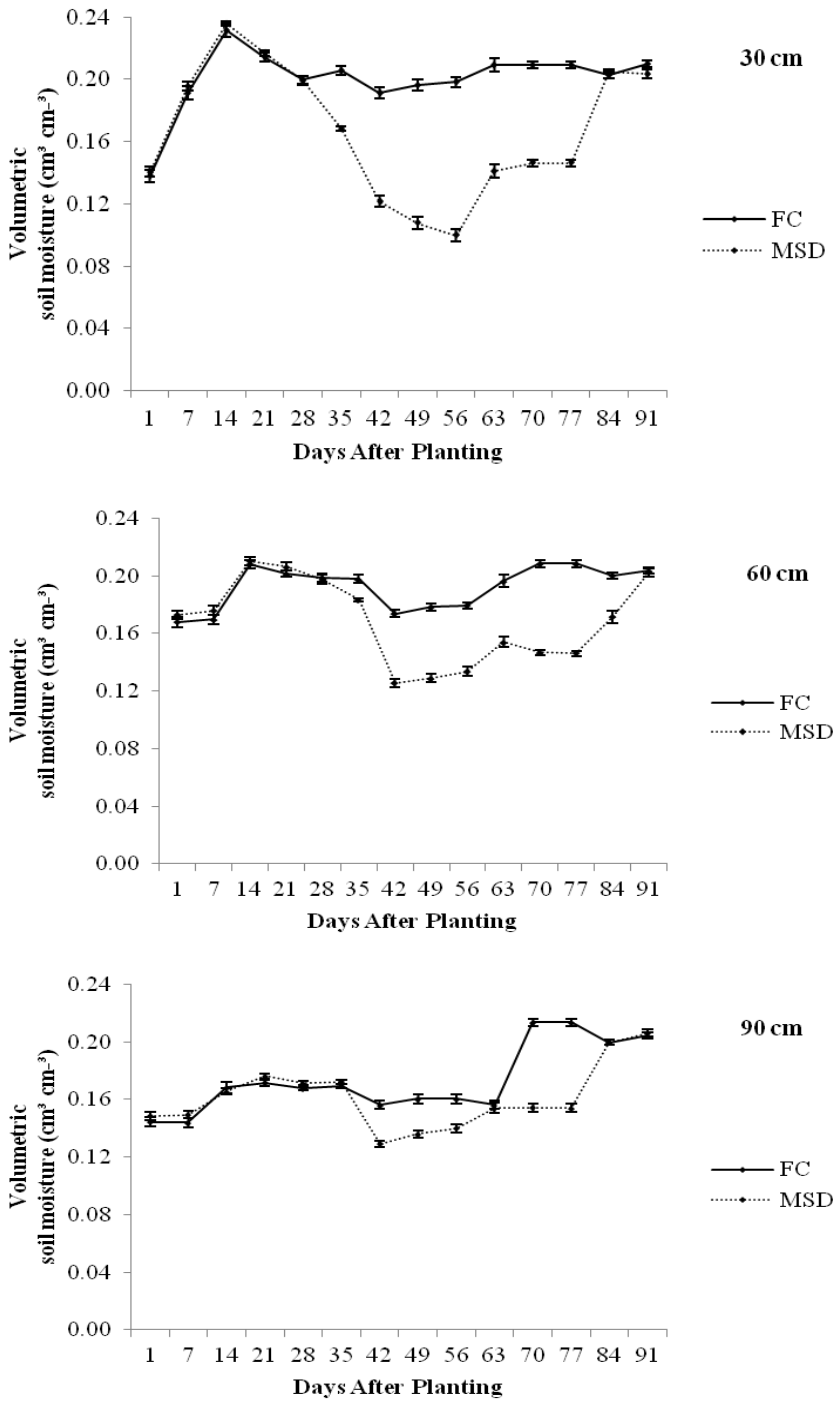

Figure 3. Volumetric soil moisture (fraction) during the crop season. Soil moisture declined at all depths during the MSD period. Data are means ± SE of FC and MSD subplots (*n* = 32 for each irrigation treatment).

The severity of the mid-season drought differed between genotypes, as judged from soil moisture depletion at 30 cm depth (P = 0.034); specifically, less soil water was lost during the MSD period in subplots planted with ICGV than with KS (P = 0.037). Less water tended to be lost from soils with inoculated compared to uninoculated plants at 30 cm depth (Table 3), although the 5C-2 had no significant effect on soil water depletion overall (P = 0.094) or on the genotypic variation (P = 0.789). The differences between treatments at 60 cm and 90 cm depths were not significant (data not shown).

Table 3. Genotypic and rhizobacterial treatment differences in soil water depletion under MSD, expressed as the soil water content remaining at 60 DAP as a percentage of the soil water content at 30 DAP. Data are means ± SE (n = 4). Across both rhizobacterial treatments, soil moisture at 30 cm depth was significantly higher within ICGV than KS subplots (according to ANOVA with a Bonferroni test).

Depth (cm)	NN		KS		KKU		ICGV	
	UC	5C-2	UC	5C-2	UC	5C-2	UC	5C-2
30	48.5 ±1.6	49.5 ±3.2	38.7 ±1.6	46.1 ±1.1	47.4 ±5.1	51.7 ±4.1	50.0 ±1.8	53.6 ±4.3
60	69.8 ±4.1	67.6 ±2.9	67.3 ±7.7	66.7 ±6.5	70.7 ±2.8	66.3 ±10.0	63.7 ±4.5	68.6 ±4.5
90	79.8 ±2.8	85.3 ±5.8	88.0 ±5.6	77.4 ±6.6	80.5 ±3.5	80.4 ±4.2	77.9 ±4.9	82.3 ±4.7

5C-2 promoted the greatest reduction in RWC caused by MSD

Genotypic differences in leaflet RWC were observed in plants at FC, and these differences were more distinct during MSD. Under MSD, the decline in RWC was considerably less, in rate and extent, for NN compared to the other genotypes. For KS and ICGV, RWC reduced most but also recovered most dramatically following re-irrigation (Figure 4).

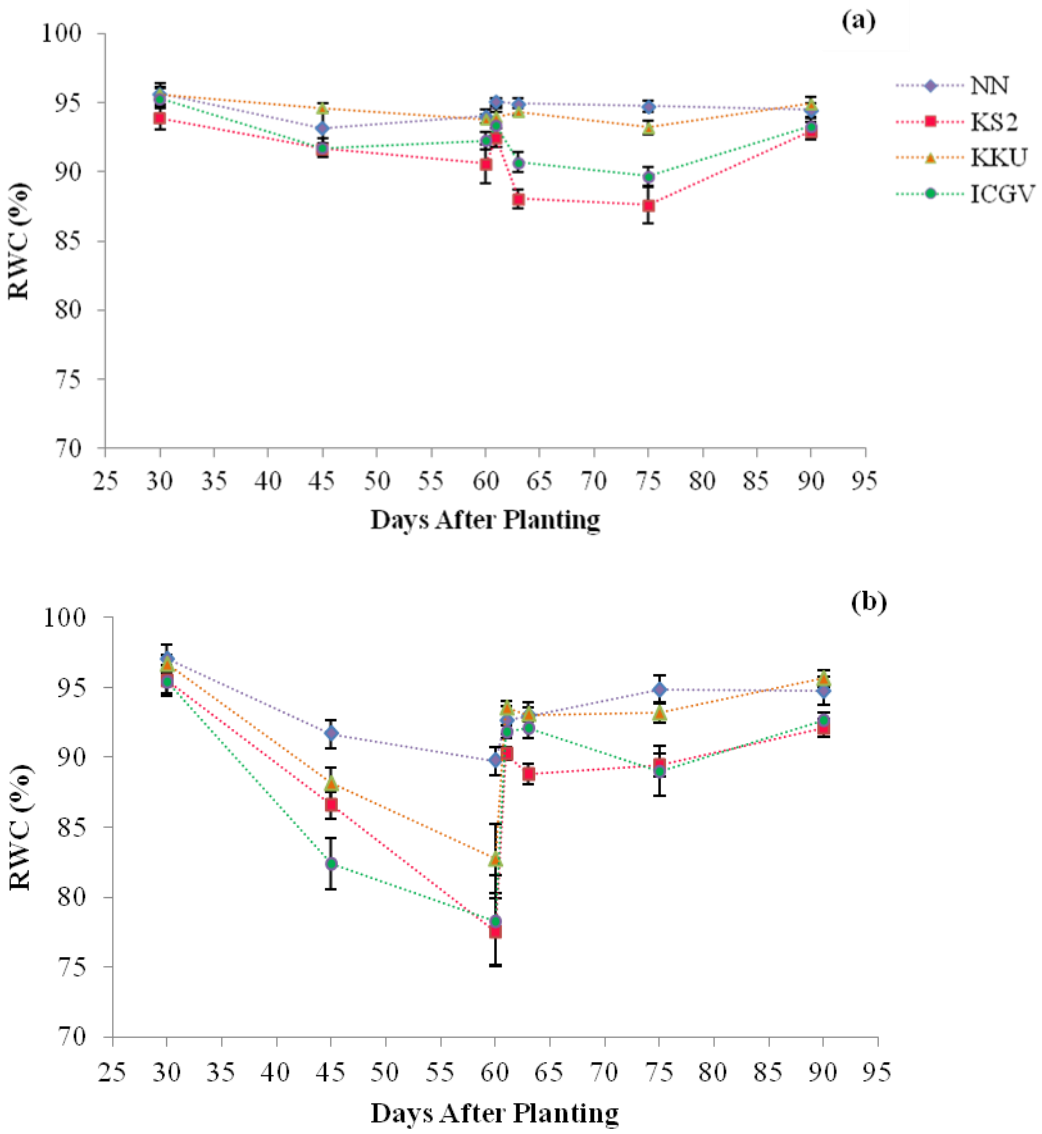

Figure 4. Changes in leaflet RWC (%) during MSD and following re-irrigation (at 60 DAP). (a) Genotypic differences in RWC for plants with soil water maintained at FC and relative stability over time; (b) genotypic differences in rates and extents of decline in RWC in plants under MSD, and increase following re-irrigation. Data are means ± SE of the UC and 5C-2 treatments combined (n = 8).

At 30 DAP, within the MSD treatment KS had lower RWC than KKU and NN (for both P = 0.03). The rhizobacterial treatment effect was not significant overall (P = 0.94) or different between genotypes (P = 0.69).

At 45 DAP, rhizobacterial effects on different genotypes varied according to irrigation (P = 0.004). For ICGV, compared to the UC the rhizobacteria

reduced the RWC under MSD (P < 0.001), but had no effect at FC. In contrast, under MSD, RWC was higher for NN than KKU but only between the UCs (P = 0.02); thus the 5C-2 negated the genotypic difference.

At 60 DAP, RWC was generally reduced by MSD, except for NN which maintained high RWC across a range of soil moisture contents (Figure 5). Under MSD, RWC was significantly higher for NN than KS (P < 0.001) and ICGV (P = 0.003), but did not differ from KKU (P = 0.31). In contrast to at 45 DAP, the rhizobacterial inoculation had no effect (P = 0.30). The MSD caused a genotypic difference in RWC, which was higher for NN than ICGV amongst the MSD plants only (P = 0.01; at FC P = 0.89).

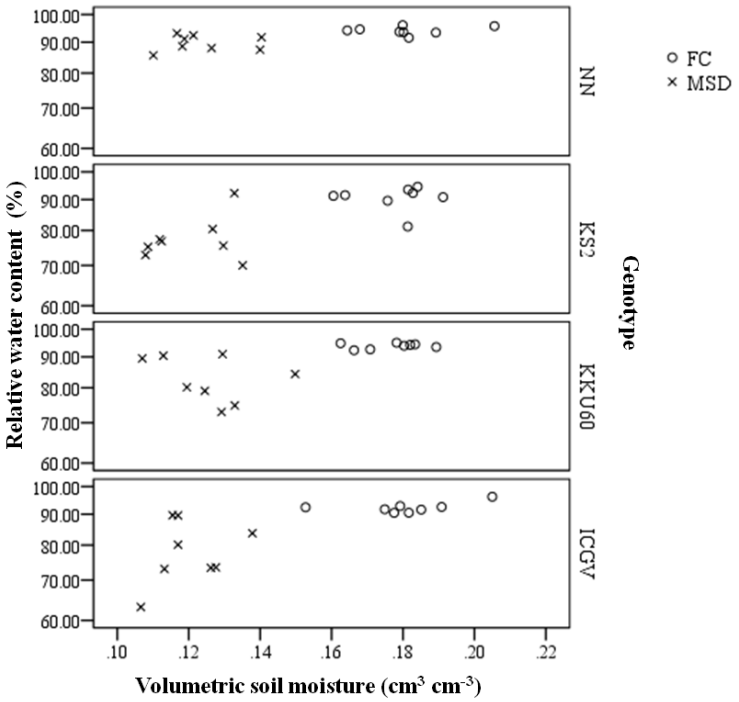

Figure 5. Leaflet relative water content (%) at different soil moisture contents (cm^3 cm^{-3}) at 60 DAP. Volumetric soil moisture (fraction) is the mean for three depths (30, 60 and 90 cm). The mean reduction in RWC for plants under MSD compared to at FC was *c.* 15 % for KS and ICGV, *c*. 12 % for KKU and *c.* 4 % for NN.

Following re-irrigation, at 61 DAP RWC was affected by irrigation (P = 0.002) and genotype (P = 0.003), whilst their interaction was not significant (P = 0.42) since RWC increased in all re-irrigated plants. At 63 DAP, RWC differed between genotypes (P = 0.02), since KKU and NN had not recovered to FC levels. The MSD ceased to influence RWC by 75 and 90 DAP (P = 0.60 and P = 0.40, respectively). The genotypic differences (P < 0.001 both times) were the same as for plants maintained at FC. The rhizobacterial

treatment did not affect the interactions between genotype and irrigation (P = 0.43, P = 0.34, P = 0.63 and P = 0.58 at 61, 63, 75 and 90 DAP, respectively).

Increased stomatal conductance with 5C-2 was negated by MSD

Stomatal conductance (g_s) was influenced by the rhizobacteria (P = 0.02) at 30 DAP, which affected g_s in different ways according to genotype (P = 0.04) despite no effect of genotype alone (P = 0.07). Specifically, inoculated KS had higher g_s than the uninoculated plants of both NN (P = 0.009) and ICGV (P = 0.02), reflecting a promoting effect of the 5C-2 for each of these three genotypes.

At both 45 and 60 DAP, g_s was significantly decreased by MSD across all genotypes and both with and without 5C-2 ($P \leq 0.001$, whereas at 30 DAP P = 0.83). At 60 DAP, g_s was significantly higher for NN than KS under MSD (P = 0.002) but did not differ in the plants at FC (P = 0.83) (Figure 6). These effects were significant between the UC and inoculated plants (data not shown). Therefore MSD abolished rhizobacterial effects on g_s.

At 90 DAP, NN had higher g_s than KS for the FC as well as rehydrated MSD plants (P = 0.02). There was no irrigation or rhizobacterial effect. Thus, re-irrigation eliminated the effects of the MSD.

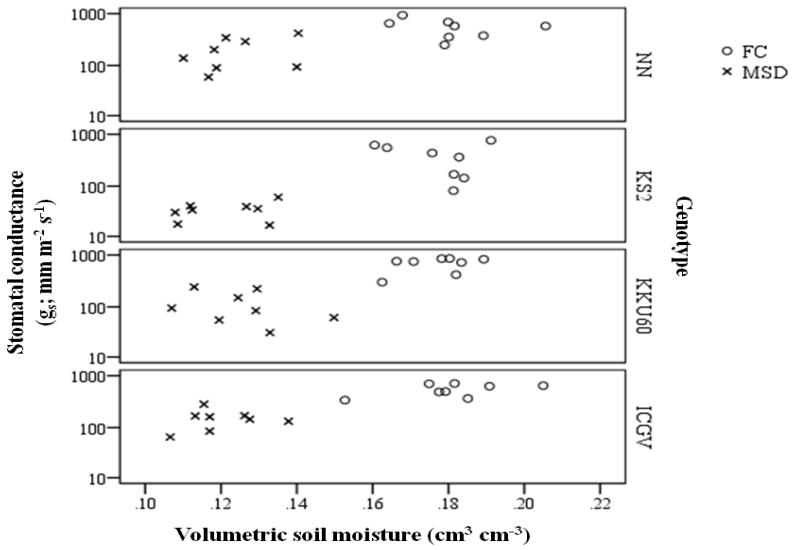

Figure 6. Stomatal conductance (mmol m^{-2} s^{-1}) at different soil moisture contents (cm^3 cm^{-3}) at 60 DAP. Volumetric soil moisture (fraction) is the mean for three depths.

Relative growth rates and absolute growth differed according to genotype and responses to the irrigation and rhizobacterial treatments, and depended on the particular growth trait (Table 4).

Table 4. Main effects of irrigation (I), genotype (G) and *V. paradoxus* 5C-2 inoculation (5C-2) and all 2- and 3-way interactions (results from ANOVA). Growth variables are: Daily leaf expansion rate (LER), relative leaf area expansion rate (RLER), leaf area (LA), relative growth rate for shoot dry weight (RGRshoot), shoot dry weight (shootDW), relative growth rate for root dry weight (RGRroot) and root dry weight (rootDW). Significant differences are: ns = not significant, $P = 0.05$ (*), $P = 0.001$ (**) and $P < 0.001$ (***). Data are for all treatments and replications ($n = 64$). Rates of expansion indicate the change during the MSD (30-60 DAP) and re-irrigation (60-90 DAP) periods, and absolute growth was measured at the end of each period.

Period	Model Term	Significance of model term on growth variable						
		LER	**RLER**	**LA**	**RGRshoot**	**shootDW**	**RGRroot**	**rootDW**
30-60 DAP	I	***	***	***	***	***	*	ns
	G	***	**	***	***	ns	*	**
	5C-2	**	ns	ns	ns	ns	ns	**
	I x G	ns	ns	*	ns	**	ns	ns
	I x 5C-2	ns	ns	ns	ns	ns	ns	ns
	G x 5C-2	*	ns	ns	ns	ns	ns	ns
	I x G x 5C-2	***	ns	ns	ns	ns	ns	ns
60-90 DAP	I	**	***	ns	***	ns	ns	ns
	G	***	**	***	ns	*	***	**
	5C-2	*	ns	ns	ns	ns	*	ns
	I x G	ns	ns	ns	*	ns	ns	ns
	I x 5C-2	ns	ns	ns	ns	ns	ns	ns
	G x 5C-2	*	ns	ns	ns	ns	ns	ns
	I x G x 5C-2	**	ns	ns	ns	ns	ns	ns

5C-2 reduced LER under MSD

For all irrigation, genotype and rhizobacterial treatments, both during and following MSD, LER (mm day^{-1}) decreased significantly as leaf length increased (Table 5). During the MSD period, irrigation, genotype and 5C-2 affected the variation in LER with leaf length ($P < 0.001$) (Table 4; Appendix 3a). Leaf growth was significantly slower under MSD than for plants at FC (Table 5; Appendix 3b).

LER was most reduced by the MSD for ICGV, an effect observed in the UC and which the 5C-2 inoculation enhanced (Table 5). Whereas the LER of uninoculated KS and KKU instead increased under MSD, the rate was reduced for the inoculated plants (Table 5). Thus the rhizobacteria either promoted (ICGV) or changed (KS and KKU) the effect of the MSD on LER, and the differences in responses were significant (Table 4). The rhizobacteria decreased LER under MSD in these three genotypes.

Re-irrigation increased LER, according to rhizobacterial inoculation

Following re-irrigation, LER from 60 to 67 DAP differed significantly between genotypes and their responses to the irrigation and rhizobacterial treatments (Table 4; Appendix 3c and 3d). Leaf length had a reduced effect on LER for the plants recovering from MSD ($P < 0.001$; Table 5; Appendices 3c and 3d). This signified enhanced LER following re-irrigation and suggested a compensatory leaf growth response of the MSD plants. This effect was most prominent for ICGV whilst not evident in uninoculated KS or KKU (Table 5), for which LER was not reduced under MSD. The genotypic differences in response to irrigation and 5C-2 were significant (Table 4).

Table 5. Regressions (r^2) with *P*-values, and regression slopes (*β*) with standard errors, for LER with leaf length as a covariate during the MSD period (37-60 DAP) and following re-irrigation (60-67 DAP), and percentage difference in the effects under MSD from FC. Lengths of expanding leaves were measured daily for two plants per subplot (*n* = 128). Data are means of eight subplots.

DAP			NN		KS		KKU		ICGV	
			UC	5C-2	UC	5C-2	UC	5C-2	UC	5C-2
37-60	FC	Regression (r^2)	0.340 (<0.001)	0.475 (<0.001)	0.328 (<0.001)	0.363 (<0.001)	0.263 (<0.001)	0.119 (0.006)	0.328 (<0.001)	0.143 (0.004)
		Regression slope (β)	-0.188 ±0.03	-0.225 ±0.03	-0.200 ±0.03	-0.192 ±0.03	-0.211 ±0.05	-0.128 ±0.05	-0.218 ±0.04	-0.136 ±0.05
	MSD	Regression (r^2)	0.373 (<0.001)	0.232 (<0.001)	0.230 (<0.001)	0.413 (<0.001)	0.294 (<0.001)	0.261 (<0.001)	0.538 (<0.001)	0.392 (<0.001)
		Regression slope (β)	-0.205 ±0.04	-0.178 ±0.04	-0.153 ±0.03	-0.257 ±0.04	-0.194 ±0.04	-0.188 ±0.04	-0.363 ±0.05	-0.315 ±0.05
		% Difference (β)	***+9.0***	***-20.9***	***-23.5***	***+33.9***	***-8.1***	***+46.9***	***+66.5***	***+131.6***
60-67	FC	Regression (r^2)	0.412 (<0.001)	0.323 (<0.001)	0.236 (<0.001)	0.289 (<0.001)	0.409 (<0.001)	0.499 (<0.001)	0.578 (<0.001)	0.422 (<0.001)
		Regression slope (β)	-0.254 ±0.02	-0.207 ±0.02	-0.239 ±0.02	-0.256 ±0.02	-0.189 ±0.02	-0.227 ±0.02	-0.359 ±0.02	-0.259 ±0.02
	MSD	Regression (r^2)	0.313 (<0.001)	0.253 (<0.001)	0.219 (<0.001)	0.297 (<0.001)	0.298 (<0.001)	0.235 (<0.001)	0.182 (<0.001)	0.167 (<0.001)
		Regression slope (β)	-0.245 ±0.03	-0.182 ±0.02	-0.254 ±0.03	-0.168 ±0.02	-0.207 ±0.03	-0.267 ±0.03	-0.216 ±0.03	-0.179 ±0.03
		% Difference (β)	***-3.5***	***-12.1***	***+6.3***	***-34.4***	***+9.5***	***+4.0***	***-39.8***	***-30.9***

5C-2 enhanced the reduction in LA under MSD

Total leaf area (LA) at 30 DAP was significantly affected by genotype ($P < 0.001$). Overall, LA of NN was lower than: KS by 36 % ($P = 0.005$), KKU by 25 % ($P < 0.001$) and ICGV by 33 % ($P < 0.001$). LA was not influenced by the rhizobacteria ($P = 0.085$).

At 60 DAP, LA was significantly reduced by MSD for three genotypes, by 23 % to 35 %, but was not affected for NN. For both KKU and ICGV, the MSD only reduced LA significantly for the inoculated plants (Figure 7). Relative leaf area expansion rates (RLER) between 30 and 60 DAP tended to be reduced most by the MSD for these genotypes (Appendix 4a), and were not affected by the rhizobacteria (Table 4).

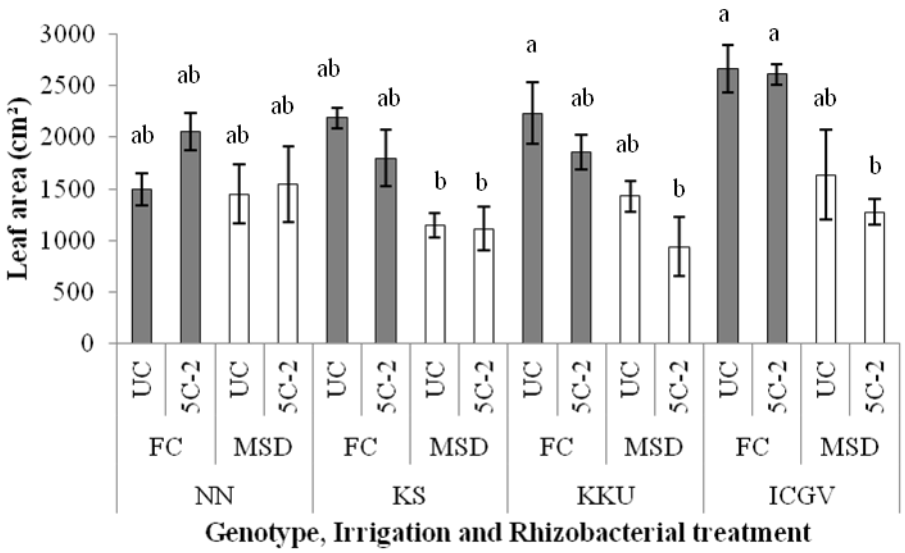

Figure 7. Whole plant leaf area (cm^2) at 60 DAP. Data are means ± SE of plants from four subplots. Different letters indicate significant differences between treatments (ANOVA, with summary statistics indicated in Table 4, and Bonferroni test; $p \leq 0.05$).

Re-irrigation recovered leaf growth

At 90 DAP, irrigation and its interaction with genotype had no effect on LA, indicating recovery from MSD. Between 60 and 90 DAP, RLER was increased by 33 % to 100 % for re-irrigated plants compared to the plants maintained at FC (Appendix 4b). This compensatory growth was greatest for KKU, for which it was most reduced under MSD. LA was higher for ICGV than all other genotypes ($P \leq 0.001$), which did not differ. In contrast to daily LER, LA and RLER were not affected by the rhizobacteria at or between 60 and 90 DAP (Table 4).

Re-irrigation recovered shoot growth

At 30 DAP, shoot DW was significantly lower for NN than all other genotypes, by 37 % to 47 %, ($p < 0.01$), and was not affected by the rhizobacteria ($P = 0.475$).

The RGR for shoot DW between 30 and 60 DAP was reduced by the MSD for all genotypes, with the greatest reduction for ICGV (99 %) (Appendix 5a). At 60 DAP, shoot DW only differed between genotypes in their responses to irrigation (Table 4), since MSD did not significantly reduce shoot biomass for NN.

Following re-irrigation, between 60 and 90 DAP shoot RGR was greater for the MSD plants. The MSD affected the increase in shoot mass differently amongst genotypes (Table 4; Appendix 5b). The greater shoot RGR in re-irrigated plants, by 67 %, was significant for ICGV ($P = 0.002$) and KKU tended to have a similar increase (70 %), whilst NN changed the least (20 %) (Appendix 5b). At 90 DAP, mean shoot DW was 11 % higher for ICGV than KKU ($P = 0.031$) but otherwise similar between genotypes; an effect that occurred independently of the irrigation and 5C-2 treatments (Table 4).

Therefore ICGV had the strongest growth increase following re-irrigation, as well as reduction under water-limited conditions. Shoot biomass recovered to FC levels for all genotypes, and NN was least responsive to irrigation.

MSD increased root expansion

Prior to the MSD treatment, at 30 DAP root DW did not differ between genotypes ($P = 0.077$) or rhizobacterial treatments ($P = 0.782$). However, root mass was lower in MSD than FC plants before irrigation was withheld ($P = 0.007$).

The MSD had the opposite effect on root growth to leaf and shoot expansion: root RGR were significantly enhanced by the MSD for all genotypes,

particularly ICGV (Tables 4 and 6). At 60 DAP, ICGV and KKU had significantly higher root DW than KS (P = 0.13 and P = 0.27, respectively). The rhizobacterial treatment did not significantly affect root expansion during the MSD (Table 4).

From 60 to 90 DAP, the MSD had no effect on root RGR or root DW, although growth rates and biomass differed between genotypes (Tables 4 and 7). At 90 DAP, root DW was highest for KKU, which was significantly higher than KS (P = 0.004). The rhizobacteria did not influence root DW at 90 DAP (Table 4).

Specific root nodulation was not reduced by MSD and did not differ between genotypes or rhizobacterial treatments, at 30 or 60 DAP (data not shown).

Table 6. Relative root DW growth rate (RGRroot) between 30 and 60 DAP. Data are means ± SE of 8 plants (with UC and 5C-2 combined, since there was no overall 5C-2 effect), from linear regression based on log-transformed root DW data vs. DAP. Percentage change as a result of MSD was calculated from the mean (RGRroot). The significance of the effects of irrigation, genotype and their interaction was assessed by ANCOVA.

NN			KS			KKU			ICGV			P values*		
FC	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**Irrigation**	**Genotype**	**Irrigation x Genotype**
0.003± 0.002	0.005± 0.002	*+66.7*	0.002± 0.002	0.006± 0.002	*+200.0*	0.004± 0.002	0.006± 0.003	*+50.0*	0.002± 0.001	0.007± 0.002	*+250.0*	0.041	<0.001	0.595

* Adjusted R^2 values for Irrigation, Genotype and Irrigation x Genotype were, respectively: 0.247, 0.323, 0.337.

Table 7. Relative root DW growth rate (RGRroot) between 60 and 90 DAP. Data are means ± SE of 8 plants and analysis was conducted exactly as for the MSD period.

NN			KS			KKU			ICGV			P values*		
FC	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**Irrigation**	**Genotype**	**Irrigation x Genotype**
0.003± 0.002	0.002± 0.002	*-33.3*	0.002± 0.001	0.003± 0.002	*+50.0*	0.004± 0.001	0.003± 0.002	*-25.0*	0.002± 0.002	0.002± 0.001	*0*	0.978	<0.001	0.994

* Adjusted R^2 values for Irrigation, Genotype and Irrigation x Genotype were, respectively: 0.086, 0.269, 0.245

DISCUSSION

V. paradoxus 5C-2 colonisation of peanut roots in a tropical field soil

Proliferation of *Variovorax paradoxus* 5C-2 was evident, with its detection on roots of all genotypes at the final assay at 37 days after inoculation.

Soil pH was within the range for optimal 5C-2 growth (R. Teijeiro, unpublished data) and also for optimal ACCd activity (Hontzeas et al., 2004; Jha et al., 2012); therefore pH was unlikely to have been limiting. For greater certainty, soil pH would need to be measured after each soil amendment application (Appendix 1).

Soil temperatures exceeded the range within which root colonisation and its physiological effects had been established for 5C-2, for different crops under various conditions. High temperatures can limit growth of mesophile bacteria, which ceased for 5C-2 at 40 °C *in vitro* (R. Teijeiro, unpublished data), although 5C-2 survival at *c.* 45 °C was confirmed (A. Belimov, pers. comm.). A temperature of 28 °C was optimal for 5C-2 growth (R. Teijeiro, unpublished data) and also for ACC deaminase activity, which was considerably decreased at 50 °C and ceased at 60 °C (Jha et al., 2012). The present study established that 5C-2 survived soil temperatures of 50 °C, recorded at the depth (5 cm) from which root samples were taken. Given the longevity of confirmed colonisation, growth can be assumed. However, the effectiveness of the ACCd enzyme in hydrolising ACC, thereby reducing ethylene, (e.g. Jha et al., 2012) was not assessed.

The maximum concentration of 10^{-6} CFU g^{-1} root FW, detected four days after the re-inoculation, was in the same range as previously determined from separate pot experiments (Belimov et al., 2009; R. Teijeiro, unpublished data). In the present study, mean concentrations were several orders of magnitude lower and declined with time, which was a more extreme reduction than reported over 78 days from pea roots in a pot trial (Belimov et al., 2009). Whilst the high temperatures did not prevent growth, further characterisation of growth rates at different temperatures would identify whether, and the extent to which, temperature reduced the rhizobacterial populations.

The rhizobacteria was applied at 10^{8} CFU ml^{-1} inoculum, which has been common amongst research with PGPR (reviewed by Lucy et al., 2004), and not lower than 5C-2 inoculations (Belimov et al., 2009; R. Teijeiro, unpublished data). Survival of rhizobacteria can be increased by applying higher concentrations of inoculum (Mawdsley and Burns, 1994), which may be required to achieve similar populations colonising roots under soil conditions that are not optimal, such as high temperatures. The lower

colonisation following the first inoculation than the second was likely due, in part, to the less direct method; therefore further exploration of application methods might enable higher concentrations colonising roots to be achieved and sustained.

The biological communities also distinguish soils from different studies with 5C-2. A degree of durability was suggested given the range of conditions under which it had been studied. Higher concentrations were not found on roots of potted plants grown in samples of field soil from which the bacterium was isolated than plants grown in a horticultural compost (Belimov et al., 2009). Although selected for its competitiveness (Belimov et al., 2005), several bacteria (also resistant to rifampicin and kanamycin sulphate) were repeatedly detected from root samples. Characterisation of the biological composition for the tropical field soil would enlighten understanding of conditions for maintaining higher populations on roots.

Genotypic differences in water relations and growth

MSD reduced shoot growth and increased root expansion

For the plants at FC, and also NN under MSD, RWC was maintained above 85%, a level which was previously reported for non-stressed peanut plants (Babu and Rao 1983).

Relative expansion rates under MSD increased for roots and decreased for leaves and total shoots, for all genotypes. A reduction in shoot growth and increase in root biomass was previously reported for peanut, for which water was withheld from 30 to 44 DAP (Furlan et al., 2012), although only one genotype was investigated. Whilst ICGV and KS had the lowest and similar RWC under MSD, their growth responses contrasted. In comparison ICGV had reduced leaf and shoot expansion rates but higher stomatal conductance and root biomass. The statement that low RWC clearly indicates a greater degree of stress in peanut (Reddy et al., 2003) did not acknowledge the possibility for plants to maintain growth due to high stomatal conductance, which is characteristic of drought tolerance (Subbarao et al., 1995), providing that the water deficit is not severe and terminal (Wilkinson and Davies, 2012; Tardieu, 2012). Although the reduction in leaf and total shoot growth under MSD might be associated with low water use efficiency (Passioura, 1977), ICGV expressed traits associated with maximizing soil water uptake and the proportion contributing to transpiration, which are central to effective water use (Blum, 2009).

The lack of difference in shoot biomass between genotypes at the end of MSD, contrasted with a previous study. Jongrungklang. et al. (2012) identified KKU and ICGV as having amongst the highest shoot biomass at

the end of soil drying and also following re-irrigation, consistently in two seasons. The previous drought occurred at a later period, with minimal soil water contents at 83 or 87 DAP, so the different timing in relation to the crop phenology imposed a different drought scenario. With the greatest impact of late-MSD being on yield components, the genotypic variation in shoot biomass was likely most affected by the peg component, due to differences in the allocation of assimilates to reproductive growth (Vorasoot et al., 2004; (Jongrungklang et al., 2012). This is typical response where water is available at depth (Tardieu, 2012).

Severity of water deficit differed according to genotype

Different levels of water deficit were experienced on a genotypic basis since soil water depletion was higher in subplots planted with KS than ICGV. Therefore ICGV had a similarly low RWC as KS despite lesser soil moisture depletion from the upper soil profile, suggesting a greater ability to extract deeper soil water. This was indicated by the previous classification of root distribution patterns, at the same field site as the present study, which revealed genotypic differences in architecture in response to late-MSD, although total biomass was not necessarily affected (Jongrungklang et al., 2012). Previously, root biomass was highest at 30 to 60 cm depth for ICGV, whereas from 0-30 cm depth for KS (Jongrungklang et al., 2012). As well as contributing to optimal growth, the high root biomass at greater soil depths, classified for ICGV and KKU under late-MSD, contributed to high yields (Jongrungklang et al., 2012).

Re-irrigation recovered leaf and shoot growth

At 90 DAP, for all genotypes the absence of an irrigation effect on LA or shoot DW indicated recovery from the reduction in growth during the MSD. Furlan et al. (2012) alternatively reported reduced shoot DW in rehydrated peanut compared to the well-watered control. Compensatory growth was indicated by the significantly increased RLER and shoot RGR in the re-irrigated plants. Whilst ICGV had the most reduced RWC, LER and shoot RGR in response to MSD, it also had the greatest recovery in response to re-irrigation. This reflected the RWC response to re-irrigation for this genotype. Although they had similar shoot REG, the higher biomass of IGGV than KKU was inconsistent with the similarly high biomass reported (Jongrungklang et al., 2012), likely due to the different timing of MSD.

The effects for ICGV likely related to its higher root expansion rate during MSD and root biomass at 60 DAP compared to other genotypes, which did not occur at 90 DAP, indicating effective water use under MSD (Blum, 2009).

Optimal benefits of increased root growth with water deficit may be achieved in scenarios with water available at depth (Tardieu, 2012), which likely describes the condition of the present study.

5C-2 effects on leaf water relations and growth

5C-2 effect on stomatal conductance was lost during MSD

Before MSD was initiated, 5C-2 promoted stomatal conductance compared to the uninoculated controls; an effect which was also shown in pea under well-watered conditions (Jiang et al., 2012). The effect on g_s was not evident after imposing MSD. Decreased stomatal conductance is a well-documented response to soil drying (Wilkinson and Davies, 2012) and the reducing effect of MSD was stronger than the promotion with 5C-2. The rhizobacterial promotion of g_s also ceased in plants maintained at FC, suggesting an influence of phenology. It was also possible that rhizobacterial populations colonizing roots may have reduced such that stomatal conductance was not affected. The increased stomatal conductance in pea reported by Jiang et al. (2012) was measured within three weeks of germination, before which the plants had been inoculated with 5C-2 (at 10^8 cells ml^{-1}) on several occasions. However, more consistent and higher populations were detected from plants under MSD than at FC plants at the end of the soil drying period. In addition, higher 5C-2 populations were detected on KS roots at the end of MSD than the start; the genotype for which the reduction in g_s was greatest. More frequent measures between 30 and 45 DAP would be needed to better understand the effects of 5C-2 on g_s in peanut.

5C-2 reduced leaf growth, promoting the MSD effect

Rhizobacterial inoculation reduced RWC for ICGV under MSD, but not plants maintained at FC. For this genotype, the rhizobacteria also enhanced the reduction in leaf expansion and leaf area caused by MSD. Rhizobacterial enhancement of the drought-induced reduction in leaf growth contrasted with previous reports of effects on shoot growth, although which did not specify leaf growth parameters. For example, shoot growth reduced less in droughted pea which was inoculated with ACCd-containing PGPR than in the uninoculated controls (Arshad et al., 2008). Pea shoot biomass increased with 5C-2, under well-watered and water-limited conditions (Belimov, 2009). Shoot RGR was also reduced to the greatest extent for ICGV, but was not

enhanced by the rhizobacteria. This further suggested a specific reduction in leaf growth in the inoculated plants.

Inoculation did not affect the greater increase in root biomass accumulation for ICGV. Thus, altered biomass partitioning was most pronounced in this genotype, and inoculation promoted this response by reducing LER and LA. The effect was not detrimental since leaf growth was recovered following re-irrigation and ICGV had also higher LA than other genotypes at 90 DAP. These results suggested that the reduced leaf area under MSD did not have a negative effect when conditions were favourable later in the crop cycle, which was contrary to the description by Tardieu (2012).

5C-2 reduced leaf growth, changing the MSD effect

Inoculation also reduced LA at 60 DAP for KKU, and LER for KS and KKU. In contrast to ICGV, the rhizobacteria changed rather than promoted the effect of the MSD, which did not reduce LER and LA in uninoculated plants. LER has frequently been reported to decrease before the detection of changes in leaf water status, such as in g_s (Sadras et al. 1992). In the present study, both parameters reduced under MSD for all genotypes, although LER depended on the rhizobacterial treatment. Consistent with ICGV, the increased root growth was not affected by inoculation. Previously, compared to fully-irrigated peanut plants, water deficit reduced LA in two drought-tolerant genotypes but not in two drought-sensitive genotypes (Vorasoot et al., 2004). KKU and ICGV have been described as having moderate and good drought tolerance, respectively (Jongrungklang et al., 2012). From the present study, the result that the reduction in LA under MSD for these genotypes was only significant in the inoculated plants, whilst not affecting the increase in root expansion, therefore suggested that the 5C-2 promoted a trait associated with drought tolerance (Vorasoot et al., 2004; Furlan et al., 2012), which was which was not significantly expressed by uninoculated plants.

5C-2 effects on growth were systemic

Rhizobacterial inoculation did not contribute to the increase in root RGR, although previous studies have identified the promotion of root growth with ACCd-utilising PGPR. For example, Dey et al. (2004) found that ACCd-containing PGPR isolates increased biomass for peanut under well-watered conditions, but did not discuss biomass partitioning. They reported increases in root length caused by inoculation under well-watered conditions. Furthermore, 5C-2 increased both root elongation and biomass well-watered and water-limited pea (Belimov et al., 2009) although only root biomass and not architecture in a different experiment, under well-watered conditions (Jiang et al., 2012). In the present study, the underestimation of total root

biomass (extracted using a monolith) was likely to have been greatest for the plants with roots at greatest depths. Thus, analysis of the effects of 5C-2 inoculation on peanut root distribution patterns (as Jongrungklang et al., 2012) would be valuable.

Nodulation was not increased for inoculated plants, in contrast to the increase reported for peanut in different ACCd-utilising PGPR strains (Dey et al., 2004), and for pea inoculated with 5C-2 (Belimov et al., 2009). The increases reported were not dependent on soil drying, so the absence of an increase in the present study was irrespective of the lack of MSD effect.

5C-2 affected LER following re-irrigation

The lack of compensatory leaf growth of uninoculated KS, whereas considerable increase in the inoculated plants, suggested that inoculation enhanced the recovery of leaf growth. Of all genotypes, KS plants likely experienced the most severe water deficit, as suggested by its higher soil water depletion at 30 cm depth and lower stomatal conductance at the end of the soil drying period. KS was also distinctive as only genotype for which the rhizobacterial population colonizing roots was higher after than before MSD.

The measures of daily LER, previously not reported for these genotypes, provided a particularly useful insight into the rate of leaf growth recovery since inoculation did not affect other measures of leaf water relations and growth. As well as changes in whole-plant phenology, the effect of leaf developmental stage on its expansion, which was suspected for soybean (Mielewczik et al., 2013), was confirmed.

CONCLUSIONS

Variovorax paradoxus 5C-2 successfully colonised roots of four peanut genotypes. It proliferated within a tropical field soil for at least 37 days after inoculation, despite maximum soil temperatures of 50°C being recorded Further investigation of soil physical, chemical and biological characteristics, which may limit population sizes colonising roots, would advance the potential to optimise benefits of 5C-2 for crop growth in different environments.

Mid-season drought (30-60 DAP) affected leaf water relations and growth differently according to genotype. Those genotypes that were more vigorous under well watered conditions (assessed at 30 DAP) had reduced leaf and shoot expansion under MSD, and increased root biomass. The reductions in leaf relative water content and stomatal conductance, and leaf and shoot growth, did not affect leaf area, leaf biomass and shoot biomass following re-irrigation, which recovered to the level of plants maintained at field capacity.

Rhizobacterial inoculation enhanced the reduction in leaf growth and LA under MSD, which was associated with high biomass after re-irrigation. Therefore *Variovorax paradoxus* 5C-2 promoted a beneficial response to MSD. Since growth recovered for all genotypes, the same biomass was achieved despite withholding irrigation for 30 days. Investigation of rhizobacterial effects on leaf water relations and growth under alternative water-limited conditions (such as terminal drought) is needed.

Key to understanding the mechanisms underlying local and systemic responses of water-limited peanut to 5C-2 would be relating the physiological and growth traits to *in vivo* ACC and ethylene concentrations, and xylem ABA concentrations (e.g. Belimov et al., 2009; Jiang et al., 2012) in roots and shoots. An interesting observation was the detection of 5C-2 on subterranean pegs and pods. The biological significance of this observation was beyond the scope of this study, but comparative ACC concentrations and possible ACCd activity could be explored further.

This study represents the first time that *Variovorax paradoxus* 5C-2 (which was isolated from Italy) has been applied to a tropical soil. Although it exacerbated the effects of mid-season drought, it promoted traits that were associated with high growth following re-irrigation. Comparing pod and seed biomass (data pending) would improve the interpretation of the effects of physiological and growth characteristics, and their significance for increasing crop yields under water-limited conditions.

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APPENDIX

Appendix 1. Timeline for crop management and phenology

The crop was sown after a fallow which was preceded by peanut cultivation.

Germination, flowering (R1) and pegging (R2) of at least 50 % of plants within a subplot was estimated by dividing the number of germinated plants within the same two rows (first and second after the border) by 38 (the number of plants in 2 rows) and multiplying by 100. Approximation of 50 % flowering was also based on observation, to reduce error if the two rows selected were not representative. Subplots were considered to have reached flowering (R1) when both estimates were at least 50 %.

DAP	Crop Management	Phenology
0	Seeds were treated with the fungicide Captan (3a,4,7,7a-tetrahydro-2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione) at a rate of 5 g kg^{-1}, and seeds of KKU 60 were also treated with Ethrel to break dormancy (48% at a concentration of 2 ml l^{-1} water).	
1 to 7	Triple superphosphate (24.7 kg P ha^{-1}), muriate of potash (KCl; 31.1 kg K ha^{-1}) and *Bradyrhizobium* (mixture of strains THA 201 and THA 205) for crop nutrition; Alachlor (2-chloro-2',6'-diethyl-N-(methoxymethyl) acetanilide 48%, w/v, emulsifiable concentrate; at 3 l ha^{-1}) to limit weeds; Acrbosulfan [2-3-dihydro-2,2-dimethylbenzofuran-7-yl (dibutylaminothio) methylcarbamate 20% (w/v), water soluble concentrate; at 2.5 l ha^{-1}], methomyl [S-methyl-N ((methylcarbamoyl)oxy) thioacetimidate 40% soluble powder; at 1 kg ha^{-1}] and carboxin [5,6-dihydro-2-methyl-1,4-oxathine-3-carboxanilide 75% wettable powder; at 1.68 kg ha^{-1}] at 1.68 kg/ha to control pests and diseases.	
5 to 12		Germination: earliest for KS and ICGV; latest related to replication and not genotype.
14	Thinning to one seedling per space	
19	Rhizobacterial inoculation (1)	
29	Rhizobacterial inoculation (2)	*[Continues]*

29	Gypsum ($CaSO_4$; 312 kg ha^{-1}) was supplied to improve pod development. Hand weeding, herbicides and pesticides were applied as needed thereafter.	
28 to 44		Flowering (R1): earliest for KKU. Occurred in 6.4 % of subplots at 30 DAP. Latest mostly due to re-planting.
45		Pegging (R2): Occurred in 78 % of subplots; significantly higher for KKU compared to other genotypes ($P < 0.001$).
59		Pod development (R3): Early development observed when root samples were taken to assay 5C-2 colonisation.
60		Pegging (R2): Occurred in 100 % of subplots
61-90		A considerable increase in flowering was observed in the MSD treatment, which was a characteristic response to re-irrigation in peanut (Reddy et al., 2003).

Appendix 2. Preparation of rhizobacterial inoculum

A growth curve for 5C-2 over 48 hours was prepared to determine the optimum time for incubation to achieve a maximum rhizobacterial concentration of the suspension, during the phase of exponential growth. Survival of rhizobacteria can be increased by applying higher concentrations of inoculum (Masdsley and Burns, 1994).

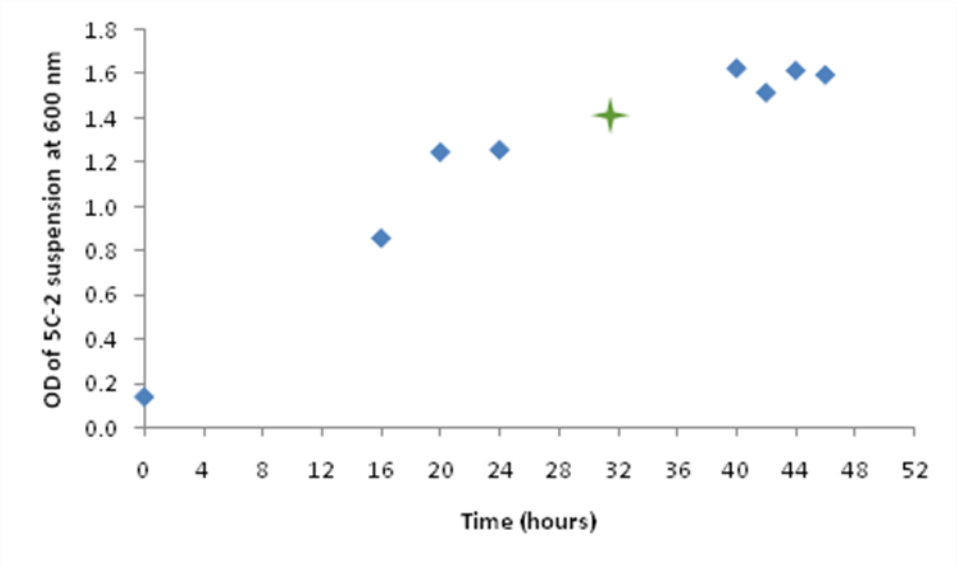

Figure 8. Optical density (OD) was calculated as absorbance multiplied by 10. The shaking incubator was set at 28°C and 180 rpm. Data are the mean of two replicates, and three absorbance readings per replicate. Measurement between 24 and 40 hours was not possible; therefore based on the curve it was extrapolated that at the mid-point, 32 hours, bacterial concentration would be an the optimum for preparation of inoculum (indicated by the green symbol).

Appendix 3. Results of ANOVA for leaf expansion rate (LER)

a.

Table 8. Contribution of genotype, irrigation and 5C-2 to the combined analysis of covariance for leaf expansion rate (mm day^{-1}) during MSD, between 37 and 60 DAP. Leaf length was used as a covariate to control for its significant effect on LER. Red text indicates the best model for predicting LER, based on highest adjusted R^2 as the goodness of fit criterion. The *P*-value indicates the significance of the added main effect or interaction.

Type	Equation for Leaf Expansion Rate (mm day -1)	*P*-value	Adjusted R^2
Main Effects	a + (Genotype)	< 0.001	0.313
	a + (Irrigation)	< 0.001	0.130
	a + (5C-2)	0.001	0.107
Main Effects	a + (Genotype) + (Irrigation)	< 0.001	0.361
	a + (Genotype) + (5C-2)	0.002	0.315
	a + (Genotype) + (Irrigation) + (5C-2)	0.001	0.362
Main Effects and Interactions	a + (Genotype) + (Irrigation) + (5C-2) + (Genotype) x (Irrigation)	0.84	0.360
	a + (Genotype) + (Irrigation) + (5C-2) + (Genotype) x (5C-2)	0.01	0.316
	a + (Genotype) + (Irrigation) + (5C-2) + (Irrigation) x (5C-2)	0.05	0.133
	a + (Genotype) + (Irrigation) + (5C-2) + (Genotype) x (Irrigation) x (5C-2)	< 0.001	0.368

b.

Table 9. Parameter estimates for final model for LER from 37 to 60 DAP: all main effects and interactions. Red text indicates the parameters which had a significant contribution to the model for LER, based on the *P*-value and 95 % confidence intervals.

Parameter	B	S.E.	*P*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	15.889	0.362	0.000	15.179	16.600
Length	-0.232	0.006	0.000	-0.243	-0.221
[Genotype=ICGV]	-1.510	0.297	0.000	-2.092	-0.927
[Genotype=KKU]	-1.335	0.298	0.000	-1.919	-0.751
[Genotype=KS]	4.605	0.294	0.000	4.029	5.181
[Genotype=NN]	0[a]	.	.	.	.
[Irrigation=MSD]	-1.209	0.306	0.000	-1.809	-0.610
[Irrigation=FC]	0[a]	.	.	.	.
[PGPR=5C-2]	-0.251	0.293	0.391	-0.825	0.323
[PGPR=UC]	0[a]	.	.	.	.
[Genotype=ICGV] * [Irrigation=MSD] * [PGPR=5C-2]	-0.081	0.600	0.893	-1.257	1.095

[Continues]

[Genotype=ICGV] * [Irrigation=MSD] * [PGPR=UC]	-0.563	0.431	0.191	-1.409	0.282
[Genotype=ICGV] * [Irrigation=FC] * [PGPR=5C-2]	0.520	0.414	0.210	-0.293	1.332
[Genotype=ICGV] * [Irrigation=FC] * [PGPR=0]	0[a]	.	.	.	.
[Genotype=KKU] * [Irrigation=MSD] * [PGPR=5C-2]	-0.589	0.598	0.325	-1.762	0.584
[Genotype=KKU] * [Irrigation=MSD] * [PGPR=UC]	0.473	0.431	0.272	-0.371	1.318
[Genotype=KKU] * [Irrigation=FC] * [PGPR=5C-2]	0.967	0.415	0.020	0.154	1.780
[Genotype=KKU] * [Irrigation=FC] * [PGPR=UC]	0[a]	.	.	.	.
[Genotype=KS] * [Irrigation=MSD] * [PGPR=5C-2]	-1.102	0.592	0.063	-2.264	0.060
[Genotype=KS] * [Irrigation=UC] * [PGPR=UC]	-0.799	0.418	0.056	-1.619	0.022
[Genotype=KS] * [Irrigation=FC] * [PGPR=5C-2]	-0.604	0.404	0.135	-1.397	0.189
[Genotype=KS] * [Irrigation=FC] * [PGPR=UC]	0[a]	.	.	.	.
[Genotype=NN] * [Irrigation=MSD] * [PGPR=5C-2]	-0.751	0.427	0.078	-1.588	0.086
[Genotype=NN] * [Irrigation=MSD] * [PGPR=UC]	0[a]	.	.	.	.
[Genotype=NN] * [Irrigation=FC] * [PGPR=5C2]	0[a]	.	.	.	.
[Genotype=NN] * [Irrigation=FC] * [PGPR=UC]	0[a]	.	.	.	.

a. Parameters set to 0 where redundant

c.

Table 10. Contribution of genotype, irrigation and 5C-2 to the combined analysis of covariance for leaf expansion rate (mm day^{-1}) during MSD, between 60 and 67 DAP. Leaf length was used as a covariate to control for its significant effect on LER. Red text indicates the best model for predicting LER, based on highest adjusted R^2 as the goodness of fit criterion. The *P*-value indicates the significance of the added main effect or interaction.

Type	Equation for Leaf Expansion Rate (mm day -1)	*P*-value	Adjusted R^2
Main Effects	a + (Genotype)	<0.001	0.294
	a + (Irrigation)	0.003	0.032
	a + (5C-2)	0.016	0.029
Main Effects	a + (Genotype) + (Irrigation)	<0.001	0.342
	a + (Genotype) + (5C-2)	0.003	0.300
	a + (Genotype) + (Irrigation) + (5C-2)	0.004	0.347
Main Effects and Interactions	a + (Genotype) + (Irrigation) + (5C-2) + (Genotype) x (Irrigation)	0.199	0.348
	a + (Genotype) + (Irrigation) + (5C-2) + (Genotype) x (5C-2)	0.036	0.351
	a + (Genotype) + (Irrigation) + (5C-2) + (Irrigation) x (5C-2)	0.483	0.346
	a + (Genotype) + (Irrigation) + (5C-2) + (Genotype) x (Irrigation) x (5C-2)	0.004	0.358

d.

Table 11. Parameter estimates for final model for LER from 37 to 60 DAP: all main effects and interactions. Red text indicates the parameters which had a significant contribution to the model for LER, based on the p-value and 95 % confidence intervals.

Parameter	B	S.E.	*P*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	12.942	0.591	0.000	11.783	14.102
Length	-0.195	0.010	0.000	-0.214	-0.176
[Genotype=ICGV]	-0.145	0.494	0.770	-1.113	0.824
[Genotype=KKU]	-1.565	0.505	0.002	-2.556	-0.573
[Genotype=KS]	5.420	0.488	0.000	4.462	6.379
[Genotype=NN]	0[a]	.		.	.
[Irrigation=MSD]	-2.187	0.526	0.000	-3.220	-1.153
[Irrigation=FC]	0[a]	.		.	.
[PGPR=5C-2]	-0.852	0.501	0.089	-1.834	0.130
[PGPR=UC]	0[a]	.		.	.
[Genotype=ICGV] * [Irrigation=MSD] * [PGPR=5C-2]	-0.171	1.006	0.865	-2.144	1.802

[Continues]

[Genotype=ICGV] * [Irrigation=MSD] * [PGPR=UC]	-0.045	0.742	0.951	-1.501	1.411
[Genotype=ICGV] * [Irrigation=FC] * [PGPR=5C-2]	-0.098	0.711	0.890	-1.494	1.298
[Genotype=ICGV] * [Irrigation=FC] * [PGPR=0]	0[a]	.		.	.
[Genotype=KKU] * [Irrigation=MSD] * [PGPR=5C-2]	2.156	1.015	0.034	0.164	4.148
[Genotype=KKU] * [Irrigation=MSD] * [PGPR=UC]	2.177	0.736	0.003	0.733	3.621
[Genotype=KKU] * [Irrigation=FC] * [PGPR=5C-2]	2.363	0.713	0.001	0.964	3.763
[Genotype=KKU] * [Irrigation=FC] * [PGPR=UC]	0[a]	.		.	.
[Genotype=KS] * [Irrigation=MSD] * [PGPR=5C-2]	0.829	0.976	0.396	-1.087	2.745
[Genotype=KS] * [Irrigation=MSD] * [PGPR=UC]	0.733	0.690	0.288	-0.620	2.087
[Genotype=KS] * [Irrigation=FC] * [PGPR=5C-2]	-0.175	0.670	0.794	-1.489	1.140
[Genotype=KS] * [Irrigation=FC] * [PGPR=UC]	0[a]	.	.	.	.
[Genotype=NN] * [Irrigation=MSD] * [PGPR=5C-2]	1.014	0.727	0.164	-0.413	2.441
[Genotype=NN] * [Irrigation=MSD] * [PGPR=UC]	0[a]	.	.	.	.

[Genotype=NN] * [Irrigation=FC] * [PGPR=5C2]	0[a]	.	.	.	.
[Genotype=NN] * [Irrigation=FC] * [PGPR=UC]	0[a]	.	.	.	.

a. Parameters set to 0 where redundant.

Appendix 4a.

Table 12. Relative leaf area expansion rate (RLER) between 30 and 60 DAP. Data are means ± SE of 8 plants (with UC and 5C-2 combined, since there was no overall 5C-2 effect), from linear regression based on log-transformed LA data vs. DAP. Percentage change as a result of MSD was calculated from the mean (RLER). The significance of the effects of irrigation, genotype and their interaction was assessed by ANCOVA.

NN			**KS**			**KKU**			**ICGV**			***P*-values***		
FC	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**Irrigation**	**Genotype**	**Irrigation x Genotype**
0.035± 0.002	0.033± 0.002	*-5.7*	0.030± 0.002	0.023± 0.002	*-23.3*	0.029± 0.002	0.019± 0.003	*-34.5*	0.033± 0.001	0.022± 0.033	*-33.3*	<0.001	0.001	0.418

* Adjusted R^2 values for Irrigation, Genotype and Irrigation * Genotype were, respectively: 0.868, 0.870, 0.885.

Appendix 4b.

Table 13. Relative LA expansion rate (RLER) between 60 and 90 DAP. Data were analysed as for the MSD period (mean± SE; n=8).

NN	**KS**	**KKU**	**ICGV**	***P*-values***

FC	MSD	% Change	FC	MSD	% Change	FC	MSD	% Change	FC	MSD	% Change	Irrigation	Genotype	Irrigation x Genotype
0.009± 0.002	0.012± 0.002	*+33.3*	0.009± 0.001	0.017± 0.002	*+88.9*	0.008± 0.001	0.016± 0.003	*+100*	0.009± 0.002	0.016± 0.002	*+43.8*	<0.001	0.002	0.134

* Adjusted R^2 values for Irrigation, Genotype and Irrigation * Genotype were, respectively: 0.668, 0.644, 0.734.

Appendix 5a.

Table 14. Shoot DW relative growth rate (RGRshoot) between 30 and 60 DAP. Data are means ± SE of 8 plants (with UC and 5C-2 combined), from linear regression based on log-transformed shoot DW data vs. DAP. Percentage change as a result of MSD was calculated from the mean (RGRshoot). The significance of the effects of irrigation, genotype and their interaction was assessed by ANCOVA.

NN			KS			KKU			ICGV			*P*-values*		
FC	MSD	% Change	FC	MSD	% Change	FC	MSD	% Change	FC	MSD	% Change	Irrigation	Genotype	Irrigation x Genotype
0.015± 0.002	0.011± 0.002	*-26.7*	0.009± 0.002	0.003± 0.002	*-66.7*	0.007± 0.002	0.002± 0.003	*-71.4*	0.012± 0.001	8.33E-5 ± 0.002	*-99.3*	<0.001	<0.001	0.342

* Adjusted R^2 values for Irrigation, Genotype and Irrigation * Genotype were, respectively: 0.373, 0.385, 0.460.

Appendix 5b.

Table 15. Shoot DW relative expansion rate (RGRshoot) between 60 and 90 DAP. Data were analysed as for MSD (mean± SE; n=8).

NN			KS			KKU			ICGV			*P*-values*		
FC	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**FC**	**MSD**	***% Change***	**Irrigation**	**Genotype**	**Irrigation x Genotype**
0.015± 0.001	0.018± 0.001	*+20.0*	0.013± 0.001	0.020± 0.002	*+53.8*	0.010± 0.002	0.017± 0.003	*+70.0*	0.012± 0.001	0.020± 0.002	*+66.7*	<0.001	0.323	0.015

* Adjusted R^2 values for Irrigation, Genotype and Irrigation * Genotype were, respectively: 0.811, 0.761, 0.825.

ACKNOWLEDGEMENTS

At the Lancaster Environment Centre:

Firstly, I am thankful to my supervisor, Dr. Ian Dodd, for providing constant inspiration, encouragement and advice.

Many thanks to Caroline Kemp for her thoughtful and patient teaching in the microbiological methods, and Shane Rothwell for his generous help with physiological techniques. I thank Carol Cook for her continuous support.

At Khon Kaen University:

Korp kun mak, mak na ka to Prof. Sanun Jogloy and Prof. Nimitr Vorasoot, for welcoming me to their research project and sharing their knowledge and interest. All members of the group enriched the experience further with their kindness, help and exuberance.

Korp kun mak na ka to Prof. Sophon Boonlue and his friendly laboratory group for their generosity and support.

I am very grateful to the Horticultural Development Company (HDC) for part-funding my Masters course.

Thank you my family, as always, for everything.