Probing roles of ethylene in leaf gas exchange, growth and development using ACC-deaminase containing rhizobacteria and *1-methylcyclopropene* (1-MCP)

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Lin Chen

BSc. Hons. Biology Sciences
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Declaration

I declare that the work presented in this thesis is my own work and has not been submitted elsewhere for the award of Doctor of philosophy degree.

Lin Chen

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Abstract

Ethylene is generally an inhibitor of plant growth and it is produced in increasing amounts when plants are exposed to abiotic stress. A number of rhizobacteria which contain the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCd) can hydrolyse ACC, the immediate precursor of ethylene, and thus decrease root ACC concentration and root ethylene evolution. Whilst promotion of plant vegetative growth by ACC-d containing rhizobacteria has been observed in different plant species, only a few studies examined the influence of this group of bacteria on plant development such as flowering time. This work presented here aims to study effects of the ACC-d containing rhizobacterium (*Variovorax. paradoxus* 5C-2) on the growth and development of *Arabidopsis,* and also investigate the role of ethylene in regulating the interactions between *V. paradoxus* 5C-2 and plants by using wild type plants and a group of ethylene related mutants. Soil inoculation with *V. paradoxus* 5C-2 promoted growth of Columbia wild type (WT) and the ethylene overproducing mutant *eto1-1*, and also enhanced floral initiation of WT plants. However, these effects were not seen in ethylene insensitive mutants (*etr1-1, ein2-1*). Soil inoculation with *V.paradoxus* 5C-2 decreased foliar ACC concentrations of wild type plants and foliar ethylene emission in both WT and *eto1-1* plants. Taken together, these results suggest *V. paradoxus* 5C-2 inoculation promotes *Arabidopsis* growth and flowering via an ethylene-dependent pathway. The effect of *V. paradoxus* 5C-2 on wheat (*Triticum aestivum cv. Ashby*) was also assessed at seedling stage (with 3 leaves), but no growth promotion was observed in wheat either in well watered or drying soil.

Further experiments investigated interactions between ABA and ethylene in stomatal regulation of wheat. Abscisic acid (ABA) is a key signal which regulates plant response to stress, particularly in regulating stomatal responses to drought. It is suggest that older leaves (3 weeks old) lose stomatal sensitivity to ABA. Recent studies indicate

Abstract

that ethylene can close stomata when ABA levels are relatively low; but antagonize ABA induced stomatal closure such that they can remain open when ABA levels increase. The work described here explored the role of ethylene in regulating stomatal responses of leaves of different growth stages to ABA, and to soil drying followed by rehydration. Furthermore, the hypothesis that lack of stomatal response to ABA in older leaves occurs via effects of leaf aging on ethylene production and/or sensitivity. Similar results as described by earlier report were obtained, whereby older, more mature leaves lost their ability to close stomata in response to exogenous ABA treatments and soil drying followed by rehydration, while young mature leaves closed stomata more fully in response to changes in water availability. Pretreating plants with 1-methylcyclopropene (1-MCP) which antagonizes ethylene receptors, or soil inoculation with rhizobacterium *V. paradoxus* 5C-2 restored the ability to close stomata after soil drying-rehydration treatments, indicating that ethylene is involved in the sluggish stomatal response to ABA in older leaves. Further work suggests that stomata of older leaves are more sensitive to ethylene compared to young leaves, explaining the relative insensitivity of stomatal closure to both ABA and drought/rehydration in older leaves. Therefore, improving stomatal response of aged leaves to soil drying via rhizobacteria or chemical (1-MCP) application can be useful to increase water use efficiency during plant vegetative growth period in agriculture practice.

Abréviations

1-MCP	1-methylcyclopropene
ABA	2-*cis*-4-*trans*-abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ACCd	ACC deaminase
ACS	S-adenosyl-L-methionine methylthioadenosine-lyase
ATP	Adenosine trphosphate
AVG	Aminoethoxyvinylglycine
CE	controlled environment
CFU	Colony forming units
GC	gas chromatography
gs	Stomatal conductance
JA	Jasmonic acid
LCOs	lipochitooligosaccharide
MAPKKK	Mitogen-activated protein kinase kinase kinase
McAb	monoclonal antibody
MS	Mass spectrometry
MTA	5'-methylthioadenosine
PAR	Photosynthetically active radiation
PBS	phosphate buffer saline
PCR	Polymerase chain reaction
PFBBr	Pentafluorobenzyl bromide
PGPR	Plant growth promoting rhizobacteria
PR	Pathogen-related genes
RIA	Radio-immunoassay
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Salicylic acid

Abréviations

S-AdoMet	S-adenosyl-L-methionine
SE	Standard error of the mean
VPD	(Saturation) vapour pressure deficit
WT	Wild-type

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Chapter1 – General introduction

1.1 Food security and drought stress

Food security is becoming an issue of global concern. Among the major challenges are an increasing human population, natural resource depletion, climate change and a shortage of good quality land for increased cropping. Although the idea of food security includes food availability, food access, and food utilization, increased food production is one of the most important parts in the food system (Renzaho and Mellor, 2010). It is estimated that the world population will increase to 9 billion by 2050. To feed this number of people, a declaration on food security targeted a 70% increase of agricultural output by 2050 (reviewed by FAO, November 2009). This declaration provides a substantial challenge to plant science and to plant scientists.

Since plants cannot move to avoid threats from the environment, they have to endure various environmental stresses which result from variable and extreme environmental conditions. These stresses include biotic and abiotic stresses that can decrease the growth or yield of plants. As water is a major component of plant tissues, the supply of water is critical for the survival of plants challenged by a dry atmosphere and by water scarcity in the soil. Water is used by plant cells to create internal hydrostatic pressures, called turgor pressure which impacts many physiological and developmental events including cell enlargement, gas exchange by the leaves, and transport in the phloem.

Plants take up water though the roots and it is evaporated from the leaves. If the amount of water uptake from the soil is less than the evaporative losses from leaves, plants show a water deficit in different aspects of their physiology processes,

especially in the reduction of leaf growth (Hsiao, 1973). By surveying agricultural productivity in the United States, Boyer highlighted the substantial negative effects of drought on crop growth, compared to other biotic or abiotic stresses. It was concluded that 41% of insurance payments for crop losses were attributable to drought (Boyer, 1982). Due to global warming, more frequent and intense droughts are being experienced in many regions of the world. There were 296 large-scale drought events (greater than 500000 km^2, and longer than 3 months) between 1950-2000 (Sheffield et al., 2009). In Asia, the area (10^6 km^2) encountering drought problems increased from 4.7 in 1982 to 8.2 in 1998. Many regions of the world (including China, Australia, part of USA, and South America) experienced serious drought in 2009 comparable to the worst recorded in human history. To meet future targets of food demand in the face of global climate change, scientists must use different technologies to explore all possibilities for improving food production in unfavorable environments.

Turner (2004) reviewed the historical trend of wheat yield in Australia from 1860 to 2000. Wheat yield nearly halved from 1860 to 1900 as soil nutrients were exhausted. From the beginning of the 20^{th} century, despite fairly constant levels of water availability, wheat yield showed a steady increase explained by improved soil management including application of superphosphate, and nitrogen fertilizers, the introduction of legumes in crop rotations, the introduction of better-adapted cultivars such as semi-dwarf cultivars, timely planting, and the use of herbicides. Therefore, yields of dry-land (rain-fed) wheat in Australia showed increased yields and rainfall use efficiency due to the use of new cultivars and new agronomic practices. It has been argued that agronomic practices account for a bigger proportion of the increase of yield in the past two decades than the use of new cultivars (Angus et al., 2001, Turner, 2004). However, some agronomic practices used

in the last 100 years have generated negative effects on the environment. Over-use of fertilizers such as P and N brings eutrophication to rivers and lakes, and also causes the dead zone in coastal due to a lack of oxygen in the water column caused by algal blooms. Soil acidification by N over-loading has become a major problem in China where intensive N fertilization has been applied (Guo et al., 2010). Global availability of phosphate for fertilizer is low (Simpson et al., 2011) and the production of nitrogen fertilizer has a high energy demand. The use of nitrogen fertilizer directly or indirectly produces greenhouse gases which partially account for the rise of average temperature on the earth (OECD, 2000). Herbicides bring a range of health and environmental issues when they are overused. Due the toxicity of herbicides, occupational exposure increases the risk of the development of Parkinson's Disease (Gorell et al., 1998) and the use of herbicides has shown negative impacts on ecology such as decreased bird populations (Blus and Henny, 1997). Environmental impacts such as these suggest that society should re-think about sustainable ways to maintain and improve crop yield but also protect the planet we live on. This thesis focuses on some novel plant science which can help us address some of the challenges highlighted above.

1.2 Root system improvement and hormone regulation

Plants take up nutrients and water though the root system to allow photosynthesis and thereby biomass accumulation. As discussed earlier, limitations in water and mineral nutrient supply will restrict the growth of plants. Yields of many crop plants show a linear relationship with increased fertilizer and water use (Loomis, 1992). In the past 100 years, higher and higher inputs of fertilizer have been applied to get high outputs of crops. However, excessive use of nitrogen fertilizer could cause growth

biomass decline by affecting apoplastic pH (Jackson, 1997, Wilkinson et al., 2007). There is evidence that crop outputs per unit fertilizer input are declining (Zhang et al., 2011). In addition to high fertilizer input, drought is one of the most critical risks that agroecosytems have to face as the result of global climate change (Easterling et al., 2000). As roots are the primary sites of water uptake by plants, a vigorous root system (early and fast root extension and proliferation, greater root biomass) may enable the plant to access more water and nutrients from the soil and may be crucial to maintain growth and yield under adverse soil conditions, particularly in the earlier stages when young plants face serious stresses (Richards, 2008, Dodd et al., 2010). A vigorous root system can intercept and capture the nutrient, particularly nitrogen uptake before it moves below the rooting depth (Wilkinson and Davies, 2002), and enhance water capture in deeper soil layers which could show the potential to significantly increase grain yield (Manschadi et al., 2006).

However, there is debate as to whether increasing the size of root system could benefit shoot growth and final yield, because the metabolic costs of root growth and maintenance can be quite substantial, which could reduce growth of photosynthetic tissues (Nielsen et al., 2001, Lynch, 2007). Earlier investment of photosynthesis products to roots to obtain more resource is particularly important in conservation farming systems (Perez-Alfocea et al., 2010). Nevertheless, under the likely future situation of less land, less water, and reduced fertilizer availability, novel methods which act to increase plant water and nutrient uptake and use efficiency could be a sustainable way to increase yield.

To increase plant nutrient and water uptake from soil and resource use efficiency, effective root traits which could improve yield potential have been used by breeders. Lynch (2007) describes a method whereby specific root traits were selected through

direct phenotypic evaluation or molecular markers rather than conventional field screening for crop yield. By focusing on root architectural traits including root growth angles, adventitious root formation and lateral branching, plants with specific traits which can enhance topsoil foraging and phosphorus acquisition from infertile soil were bred (Lynch, 2007). Crossing different genotypes to develop inbred lines combined new traits including both increased shallow roots and deep roots in common bean and soybean cultivars, thereby increasing phosphorus efficiency (shallow roots) and drought tolerance (deep roots) in the new genotype (Beebe et al., 1997, Bonser et al., 1996, Liao et al., 2006, Liao et al., 2001). A new trait with abundant root cortical aerenchyma (RCA) can reduce root metabolic costs, thereby permitting greater root growth and water acquisition from drying soil, hence increasing maize drought tolerance compared to the performance of a genotype with low RCA (Zhu et al., 2010).

In addition to specific root trait selections, rhizosphere engineering with micro-organisms is another low-cost option to apply in the field to improve plant nutrient and water use efficiency. By taking advantage of mycorrhizal symbioses which occur in the majority of higher plant species, ectomycorrhizas and arbuscular mycorrhizas have been used to enhance phosphorus acquisition by plants and (Smith and Gianinazzi-Pearson, 1988, Smith and Smith, 1990, Smith et al., 2003). Other research employs rhizobacteria to regulate root-shoot hormone balance, thereby increasing water use efficiency of several plants such as peas and potatoes (Belimov et al., 2009 b, Belimov et al., 2009 a, Glick et al., 1995, Glick et al., 1997). Root and shoot biomass of pea experiencing water deficit can be increased by application of the ACC deaminase-containing rhizobacterium *Variovorax paradoxus* 5C-2 which can break down ethylene precursor ACC and hence regulate ethylene accumulation in roots and shoots (Belimov et al., 2009 b). As ethylene is known as 'stress hormone'

and negatively regulates several aspects of plant growth such as primary roots under low water potential (Spollen et al., 2000), and shoot growth (Sharp, 2002, Pierik et al., 2006). Regulation of ethylene production under stresses could be one of the approaches to improve growth of plant with no more input in the agriculture system.

1.3 Ethylene signalling

The plant hormone ethylene is a gaseous hormone with simple two-carbon structure. However, ethylene is also involved in a variety of plant developmental events (such as seed germination, flowering, senescence of leaves and flowers, root growth and development, fruit ripening, and sex determination) (reviewed by Ecker, 1995) and responses to external stimuli. More importantly, ethylene is also recognized as a stress hormone because its production is induced by a number of stress signals, including pathogen infection, mechanical wounding, chemicals and metals, ozone, drought, and extreme temperatures (Kende, 1993, Ecker, 1995).

1.3.1 Ethylene biosynthesis

The precursor of ethylene biosynthesis is S-adenosyl-L-methionine (S-AdoMet) (Yang and Hoffman, 1984). S-AdoMet synthetase (SAM synthetase, EC 2.5.1.6) can convert nearly 80% of cellular methionine to S-AdoMet by utilizing ATP (Giovanelli et al., 1985). As well as being the substrate of ethylene biosynthesis, S-AdoMet is also the major methyl-group donor in plants and is involved in polyamine biosynthesis, and the modification of lipids or proteins or nucleic acids (Ravanel et al., 1998). The first step of ethylene biosynthesis in the Yang cycle is the conversion of S-AdoMet to ACC that is catalyzed by ACC synthase (ACS) (S-adenosyl-L-methionine methylthioadenosine-lyase, EC4. 4. 14) (Kende, 1993, Yang and Hoffman, 1984). The

enzyme ACC oxidase converts ACC to ethylene, carbon dioxide, and cyanide, which is detoxified to β-cyanoalanine to prevent cyanide accumulation. In the reaction, oxygen is an essential factor and at the same time, Fe^{2+} and ascorbate are required to work as cofactor and co-substrate, respectively. In addition, ACC synthase also produces 5'-methylthioadenosine (MTA), which is converted to methionine though a modified methionine cycle (Wang, Li et al. 2002). In this reaction, the methyl group is preserved for another round of ethylene production. Therefore, the pool of methionine is maintained and ethylene can be synthesized continuously (Wang et al., 2002). The rate limiting step of ethylene synthesis is the conversion of S-AdoMet to ACC by the enzyme ACC synthase.

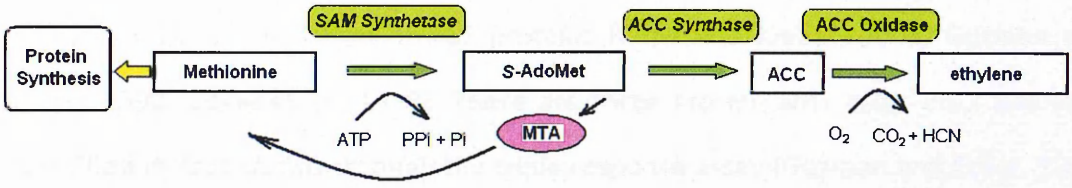

Figure1.1. Biosynthesis pathway of ethylene

Recent studies suggest ACC synthases are encoded by multigene families in all species examined and are highly regulated by multiple internal and external signals. There are 12 *ACS*-like genes in the *Arabidopsis* genome and 7 *ACS* genes have been identified and characterized (Arteca and Arteca, 1999, Liang et al., 1992, Samach et al., 2000, Yamagami et al., 2003). Many studies have addressed the spatial and temporal regulation of these *ACS* gene activities by various endogenous cues (Vanderstraeten et al., 1992) and environmental stimuli (Wang et al., 2002). Indole acetic acid (IAA) induced gene expression of all *ACS* except *ACS7* and *ACS9* genes in root tissues (Yamagami et al., 2003). Meanwhile, IAA treatment can extend the expression of the *ACS7* gene from the vascular zone to a layer of the parenchymatous tissue

(Tsuchisaka and Theologis, 2004). Ethylene can induce the expression of *ACS2* and *ACS6* genes in the mature leaves (Vanderstraeten et al., 1992). Cytokinin up-regulated the expression of the *ACS5* gene (Vogel et al., 1998). *ACS6* is induced by touch in the leaves of mature plants and also by wounding (Arteca and Arteca, 1999). *ACS4* gene expression is also regulated by wounding (Abeles, 1992, Liang et al., 1996). Expression of *ACS6*, *ACS8* and *ACS9* genes is increased in *Arabidopsis* rosettes by reducing the light intensity (Vandenbussche et al., 2003).

In addition to the regulation of ethylene production by transcription increasing or decreasing *ACS* gene expression, posttranslational regulation of ACS protein activity is another important mechanism in controlling ethylene biosynthesis. Studies of ethylene overproducing (*eto*) mutants in *Arabidopsis* reveal the possibility of posttranslational regulation of ACS proteins (Chae and Kieber, 2005, Guzman and Ecker, 1990, Vogel et al., 1998). There are three *eto* mutants *eto1*, *eto2* and *eto3* identified in *Arabidopsis* through the triple response assay (Guzman and Ecker, 1990, Kieber et al., 1993). In *Arabidopsis*, the triple response is characterized by the inhibition of hypocotyl growth and root elongation, a thickening of the hypocotyl and an exaggerated apical hook (Chen et al., 2005). *eto* mutant seedlings show an ethylene response phenotype under ambient air and 10- to 40-fold more ethylene is produced from *eto* seedlings than wild type seedlings grown in the dark. *eto1* is a recessive mutant and *eto2* and *eto3* are dominant. The protein ETO1 has been identified as an E3 ligase component, a BTB/TPR protein (Wang et al., 2004). It negatively regulates ACS5 protein activity by directly interacting with the full-length protein of ACS5 which is degraded rapidly in wild-type etiolated *Arabidopsis* seedlings *in vitro*, this interaction can be disrupted by the *eto2* mutation. *In vivo* analysis suggested the stability of ACS5 protein was increased in the *eto1* mutant (Chae et al., 2003). The *eto3* mutant phenotype is the result of a s-sense mutation-V457D within

the C-terminal of ACS9 protein, which is the closest homolog of ACS5 protein in the *Arabidopsis* genome (Chae et al., 2003). The eto2 mutation is a single base-pair insertion of the C-terminus of the ACS5 protein by slowing down ACS5 protein degradation (Vogel et al., 1998). These results indicate that the phenotypes of *eto* mutants are gained by decreasing the rate of ACS protein degradation, which is an important mechanism to control ethylene production. However, the mechanism of ACS protein degradation *in vivo* is still not well understood.

In contrast to *ACS* genes and proteins, studies in tomato suggest that ACC oxidase (ACO) is constitutively present in most tissues and its expression increases during fruit ripening (Ecker, 1995). Since the synthesis of ACC is the key step to control ethylene production, mechanisms of *ACO* gene regulation have been less discussed in the literature. Recently studies of ethylene synthesis have addressed how endogenous and external signals govern the differential expression of *ACS* and *ACO* genes/enzymes in various plant species. However, many important questions about regulatory mechanisms still remain. For example, how do hormones like cytokinins, and biotic or abiotic stresses like wounding or pathogen attack evoke rapid ethylene biosynthesis from plant cells? Given the large number of *ACS* and *ACO* gene isoforms identified after the completion of the *Arabidopsis* genome sequence, it is necessary to ask why plants need multi-gene families for ethylene biosynthesis? Do these proteins have equivalent biochemical activities and similar regulatory mechanisms? Future research focused on the biochemical regulation of ACS/ACO proteins and the components involved in this regulation could further the understanding this 'stress induced' hormone-ethylene.

1.3.2 Ethylene signalling pathway

After ethylene synthesis from S-AdoMet, it is perceived by several receptors which

invoke transfer through an intracellular signalling pathway, to trigger specific biological responses. Many key components of the ethylene signal transduction pathway including the receptors have been identified on the basis of the highly reproducible triple response in dark-grown *Arabidopsis* seedlings. Cloning and characterization of these genes are generating a clearer picture of the ethylene signal transduction pathway.

1.3.2.1 Ethylene perception

In *Arabidopsis*, ethylene is perceived by a family of five membrane-localized receptors (ETR1, ETR2, ERS1, ERS2, EIN4 proteins) that are homologous to two-component histidine kinases from bacteria (Chang et al., 1993, Hua et al., 1995, Hua and Meyerowitz, 1998, Hua et al., 1998, Sakai et al., 1998). Among these receptors, ETR1, ETR2 and EIN4 proteins have both a putative histidine protein kinase and a receiver domain, whereas, ERS1 and ERS2 proteins lack this receiver domain. The receptor family can be divided into two groups depending on structural similarities of the sensor domain: the ETR1-like subfamily including ETR1 and ERS1 proteins can form a membrane-associated, disulfide-linked dimer and contain ethylene-binding sites at the N-terminal region (Hall et al., 2000, Schaller et al., 1995); the ETR2-like subfamily, consisting of ETR2, EIN4 and ERS2, have four hydrophobic sub-domains at the N-terminus and a degenerate histidine kinase domain, which are considered necessary for catalytic activity (Hua et al., 1998, Sakai et al., 1998). Loss of function mutations in any single ethylene receptor show little or no effect on seedling growth in the absence of ethylene and this suggests there is functional overlap within the receptor family (Hua and Meyerowitz, 1998). Quadruple mutations (mutation of *ETR1*, *ETR2*, *EIN4* and *ERS2* genes) in the receptors show constitutive ethylene responses, indicating that these receptors negatively regulate ethylene responses.

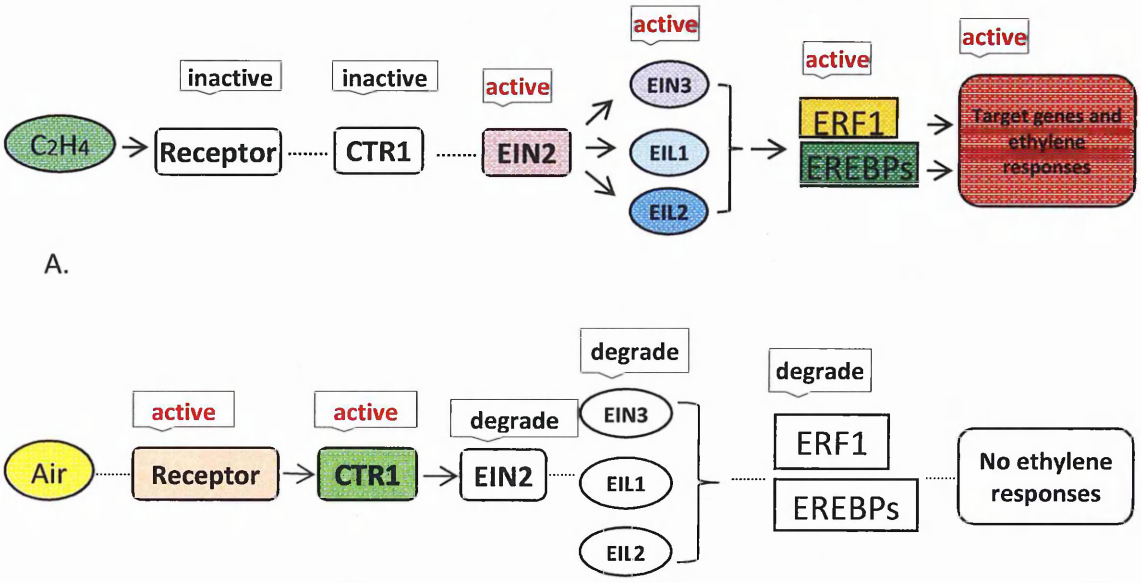

Figure1. 2. Ethylene sigalling pathway

A. Binding of ethylene inactivate receptors, resulting in deactivation of CTR1, which allows EIN2 to function as a positive regulator of the ethylene pathway. EIN2 positive signals relieve the repression on EIN3/EIL transcription factors and ethylene responses.

B. In the absence of ethylene, ethylene receptors activate CTR1, and CTR1 in turn negatively regulates the downstream ethylene response pathway.

1.3.2.2 Ethylene Signalling

Several ethylene mutants were identified through the triple response assay. The *ctr1* mutant shows a dwarf phenotype typical of ethylene treated plants, indicating its negative role in the ethylene signalling pathway (Kieber et al., 1993). Loss of function mutations in *EIN2* gene cause complete ethylene insensitivity for all ethylene responses tested throughout plant development, revealing that EIN2 protein is an essential positive regulator in the ethylene pathway (Alonso et al., 1999, Roman et al., 1995). *ein3* mutants show a loss of ethylene-mediated response and these can be rescued by over-expression of *EIN3*, *EIN3-like (EIL)1* or *EIL2* gene in this mutant,

indicating that they all mediate the ethylene response (Chao et al., 1997). These findings strongly contribute to our understanding of ethylene signalling downstream of reception.

The ethylene signaling pathway is regulated by both positive and negative signal components. Without ethylene in the air, ethylene receptors activate the kinase activity of CTR1 which is identified as a member of the Raf-like ser/thr kinase family with similar characteristics to mitogen-activated protein kinase kinase kinase (MAPKKK) (Huang et al., 2003, Kyriakis et al., 1992). *CTR1* gene is a negative regulator that actively suppresses the downstream ethylene response gene, such as EIN2 gene which encodes a novel integral membrane protein and the EIN3/EIL transcription factors which act downstream of EIN2 in the absence of ethylene. Ethylene binds the receptors and makes the CTR1 protein inactive (Alonso et al., 1999, Roman et al., 1995). Therefore CTR1 protein no longer suppresses the signal pathway and this activates EIN2 protein, inducing the signal cascades.

Other experimental data suggest that the basic components and mechanisms of the ethylene signal transduction pathway are conserved in most species including dicotyledons and monocotyledons, although some differences exist (Klee, 2004). Ethylene receptors have been identified in many other plant species, including rice (*Oryza sativa.*), tobacco (*Nicotiana tabacum L.*), cucumber (*Cucumis sativus L.*), and tomato (*Solanum lycopersicum*) (Klee, 2002, Terajima et al., 2001, Yau et al., 2004). The presumed structures of the tomato receptor family are very close to those in *Arabidopsis* and each tomato receptor gene is expressed differently throughout plant development stages and in response to different environmental stresses (Klee, 2002). *LeETR1* and *LeETR2* genes are constitutively expressed in all tissues of tomato throughout the life cycle, but *LeETR1* gene expression level is 5 times higher than

LeETR2. These two genes didn't show any altered expression in response to external stimuli such as ethylene or pathogen infection. *NR* and *LeETR4* genes can be induced by pathogen infection. Although many ethylene receptors have been identified in various plant species, less information is available for other downstream components in the ethylene signalling pathway such as *CTR1*, *EIN2* and *EIN3/EILs* genes in other plant species. There are three *CTR1*-like genes found in tomato and two genes *EIN2*, *EIL* identified in maize (*Zea mays*) (Adams-Phillips et al., 2004, Gallie and Young, 2004). But the components involved in the ethylene signalling pathway are still not clear in plant species other than *Arabidopsis*. Some questions such as whether other species share a similar ethylene signalling pathway to that in *Arabidopsis* and whether regulation mechanisms are also conserved in different species still remain to be answered.

1.3.3 The Involvement of Ethylene in Various Plant Stress Responses

Ethylene is involved in plants response to biotic and abiotic stresses (Wang et al., 2002) and regulation of ethylene to plant responses could include different aspects, for example in the plant resistance to disease, ethylene is involved in regulation of symptom development or cell death in pathogen infection, defense gene expression and interacting with other signaling such as the jasmonic acid (JA) and salicylic acid (SA) signalling pathways (Wang et al., 2002, Ecker, 1995). Abiotic stress-induced ethylene production such as high temperature, drought, and ozone can cause growth inhibition, and yield loss (Djanaguiraman and Prasad, 2010, Hays et al., 2007, Sharp, 2002, Zhang et al., 2009). Interactions between ethylene and other hormone signaling such as ABA are also important in regulating plant responses to abiotic stresses (Sharp et al., 2000, Wilkinson and Davies, 2010, Zhang et al., 2009).

1.3.3.1 Ethylene in biotic stress response

The sensitivity of plants to ethylene is an important variable which impacts on the susceptibility of plants to disease and insect (Ecker, 1995, Adie et al., 2007). The ethylene-insensitive mutant *ein2* of *Arabidopsis* showed less disease symptoms than the wild type when plants were infected by virulent *Pseudomomas. syringae* pv *tomato* or *Xanthomonas campestris* pv. *campestris* (Bent et al., 1992). It is also suggested that ethylene can function both in plant-herbivore and plant-plant communication, particularly function synergisticly with JA or even SA (Adie et al., 2007)Further studies show that the ethylene signalling pathway regulates expression of a group of pathogen-related (*PR*) genes which are related to the disease resistance by coordinating with the JA pathway. Moreover, variable cross-talk between JA/ethylene- and SA-dependent pathways is important in the systemic acquired resistance which triggers a long-lasting plant response against subsequent infections by pathogens (Ecker, 1995, Ryals et al., 1994).

1.3.3.2 Ethylene and abiotic stress response

Ethylene, wounding and ozone

It is well known that wounding stimulates biosynthesis of ethylene, through the induction of ACS activity (Kende, 1993). The stimulation of ethylene production has been shown to potentiate JA action in the wound response by co-regulation of the expression of proteinase inhibitor gene (*PIN II*) which are specific molecular markers for wound response with JA (Odonnell et al., 1996). By using *ACO* antisense transgenic lines, as well as inhibitors of ethylene biosynthesis or perception, it was demonstrated that induction of *PIN II* expression required active ethylene signaling in wounded tomato plants. However, direct application of ethylene did not induce *PIN II* expression, indicating that synergistic effects between ethylene and JA signaling are

important in regulating expression of wounding response genes (Odonnell et al., 1996).

Apart from wounding, ozone rapidly stimulates ethylene biosynthesis (Vahala et al., 1998, Overmyer et al., 2000). Stimulation of ethylene production preceded synthesizes of JA and salicylic acid (SA) which both are involved in ozone response (Vahala et al., 1998). In *Arabidopsis*, ozone exposure stimulated ACS6 activity within 30 min, and the production of ethylene reached the maximal rate in an hour, and then gradually declined (Vahala et al., 1998). Further work on SA and JA pathway mutants suggest that ethylene signaling is required for cell death and it acted synergistically with SA signal pathway but is antagonized by JA pathway (Wang et al., 2002).

Ethylene, high temperature and drought

High temperature promoted ethylene production in different plant species, such as tomato (Lurie et al., 1996), pepper (Aloni et al., 1995), lettuce (Qin et al., 2007), and wheat (Hays et al., 2007). The high temperature induced ethylene can cause different problems to the plants, including cause premature leaf senescence in soybean (Djanaguiraman and Prasad, 2010), pepper flower abscission (Aloni et al., 1995), and kernel abortion in developing wheat grains (Hays et al., 2007). Work on tomato suggested high temperature stimulated ethylene production though expression of *ACC* oxidase (Lurie et al., 1996). It is suggested ethylene can enhance oxidative stress caused by high temperature by decreasing antioxidant defenses of plants (Munne-Bosch et al., 2004). High temperature stress usually is companied by drought in the field. However, compared to the effects of high temperature on ethylene production, the effect of drought is still under debate (Morgan and Drew, 1997, Wilkinson and Davies, 2010).

Low tissue water potential can increase ethylene production in detached leaves (Aharoni et al., 1979, Apelbaum and Yang, 1981, Morgan et al., 1990), but not in intact plants (Morgan et al., 1990, Narayana et al., 1991, Eklund et al., 1992). Morgan & Drew (1997) concluded that rapid desiccation of detached leaves promoted ethylene biosynthesis, while soil drying did not increase ethylene synthesis of intact plants. Other factors including increased soil compaction, the reduction of N availability, and increased of soil salinity, contribute to an increase in ethylene production. Hussain et al. (1999) demonstrated that increases in soil compaction promoted ethylene production and decreased leaf expansion rate, but these responses were not found in $ACO1_{AS}$ transgenic plants. Furthermore, by using split-pot approaches Sobeih et al. (2004) observed that ethylene production in tomato leaves was increased by partial soil drying and this was accompanied by reduction in leaf growth of wild type plants. The low-ethylene producing transgenic plant $ACO1_{AS}$ did not show increased ethylene production or leaf growth reduction in response to partial soil drying. Another view proposed by Gomez-Cadenas (1996) suggested that soil drying induced root accumulation of the ethylene precursor ACC. Re-watering induced a pulse of ACC transport to the shoot and then increased ethylene production of shoots. This ethylene peak decreased several hours later after re-watering.

As roots are the site of nutrient and water uptake, maintenance and improving of the root growth and health, particularly under stress conditions, are critical to maintain shoot growth. Several techniques which improve root traits or root behaviors have been tested including soil management and genetic work. Usage of a group of plant beneficial rhizobacteria (plant growth promoting rhizobacteria-PGPR) is one of approaches to improve root growth and health, thus improve shoot growth as it is discussed early (Lugtenberg and Kamilova, 2009, Vessey, 2003, van Loon, 2007).

These beneficial rhizobacteria (with consideration of non-phytopathogenic characteristic of strains) offer several benefits to agriculture including a lost cost, low environmental impact and suitability for application in agriculture in developing societies.

1.4 Introduction of Plant Growth Promoting Rhizobacteria

The rhizosphere is the narrow region of soil where intense interactions among plant, bacterial, and fungal partners occur (L., 1904). The rhizosphere has up to a 100-fold greater population density of bacteria than the bulk soil without plant roots and nearly 15% of the root surface may be covered by a number of bacterial species. Plant roots secrete exudates such as amino acids and sugars which provide a rich source of energy and nutrients to bacteria, leading to greater bacterial populations in the rhizosphere (Gray and Smith, 2005, Lugtenberg and Kamilova, 2009). In this area, some bacteria associated with plants roots are able to stimulate plants growth by different mechanisms and are referred as plant growth promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009, Vessey, 2003, van Loon, 2007, Glick, 1995). Some PGPR can promote plant growth indirectly by inducing resistance to pathogens or preventing the deleterious effects of one or more phytopathogenic organisms which inhibit plant growth and development (Kloepper et al., 1992) . Some PGPR can directly promote plant growth by producing compounds such as plant hormones or enhancing plant nutrient uptake (Arshad and Frankenberger, 1991, Vessey, 2003, Dodd et al., 2010). Sometimes, a single bacterial strain can provide multiple beneficial effects to plants.

One of the widely-studied example of PGPR is the *Rhizobium*-legume symbiosis (Gray and Smith, 2005). In this model, *Rhizobium* bacteria attach to plant root hairs and secrete a host-specific lipochitooligosaccharide (LCOs) -Nod factor to induce root nodule development. Within the nodules, *Rhizobium* bacteria grow by taking carbohydrates from the host and providing fixed nitrogen in return. In nitrogen-poor conditions, *Rhizobium* bacteria can promote legume plant growth by providing this important nutrient for amino acid biosynthesis of plants.

Some PGPR can enhance the solubilization of minerals such as phosphorus and iron from soil to be taken up by plant roots (Kloepper et al., 1980, Neilands, 1982). PGPR such as *P. putida* and *P. aerruginosa* can produce and secrete siderophores that are iron-chelating agents with a high affinity for Fe^{3+}, and this iron-siderophore complex can be taken up by plant roots. Later the iron is released from the siderophore and used by the plant tissues (Crowley et al., 1988). At the same time, siderophores also decrease the Fe^{3+} levels of the rhizosphere which are crucial for the survival of some fungal pathogens like *Fusarium oxysporum* and *Pythium spp.*.

The production of phytohormones including IAA (indole-3-acetic acid), cytokinin and gibberellin or other compounds by bacteria is another common mechanism by which PGPR affect plant growth and development (de Salamone et al., 2001, Dodd et al., 2010, Loper and Schroth, 1986). Studies suggests up to 80% of rhizobacteria can produce IAA (Loper and Schroth, 1986). Some *Pseudomonas fluorescens* strains show the ability to promote seedling emergence and increase plant root length by producing cytokinins (de Salamone et al., 2001). *Bacillus pumilus* and *B. licheniformis* produce high levels of gibberellins: A_1, A_3, A_4 and A_{20} and promote plant stem and shoot elongation in *Alnus. glutionsa* (Gutierrez-Manero et al., 2001, Manero et al., 1996, Ramos Solano et al., 2008). Rather than exuding phytohormone signals to the

rhizosphere, a group of PGPR can utilize phytohormones exuded from plant roots and indirectly affect plant hormone concentrations.

1.4.1 ACC deaminase containing PGPR

A number of PGPRs contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can hydrolyse ACC (the immediate precursor of the plant hormone ethylene) to ammonia and α -ketobutyrate. These bacteria take up ACC from plant root exudates and use ACC as a sole nitrogen source (Jacobson et al., 1994, Glick et al., 1995). This may lead to a decrease of plant root ACC concentration (Penrose et al., 2001) and root ethylene evolution (Madhaiyan et al., 2006), and thus increase root growth (Glick et al., 1998, Belimov et al., 2001). However, the mechanism of bacterial uptake of ACC from plants is still not well understood.

A proposed model of the interaction between ACC-deaminase-containing PGPR and plants suggests that bacteria bind to plant seeds or roots in the soil, and synthesize and secrete IAA (Glick et al., 1998). In this model, plants take up the IAA produced by bacteria and IAA can stimulate activity of the enzyme ACC synthase, and thus increase plant ACC production. Bacteria take up ACC exuded from plant seeds or roots and thus decrease their internal ACC accumulation. Although many different PGPR ACC-deaminase containing strains which belong to different families can promote plant growth (Belimov et al., 2009 a, Belimov et al., 2009b, Belimov et al., 2007, Belimov et al., 2005, Glick et al., 1995, Madhaiyan et al., 2006), and these bacteria decrease ACC concentration in the root (Penrose et al., 2001), xylem sap (Belimov et al., 2009 b) and ethylene accumulation in the whole seedlings (Mayak et al., 2004a, Mayak et al., 2004b), an essential role of IAA in this model has not yet been directly

demonstrated. For example, *Alcaligenes xylosoxidans* which contains ACC-deaminase but cannot produce IAA *in vitro* promoted growth of rape seedlings (Belimov et al., 2001).

PGPRs containing ACC deaminase have been used to maintain or even promote growth of plants under different stress conditions, which indicates the potential use of PGPR in agricultural practice. Strain *Achromobacter piechaudii* AVR8 which contains ACC deaminase significantly increased the fresh and dry weights of tomato seedlings under salt stress (Mayak et al., 2004a). Ethylene overproduction induced by salt stress was decreased by inoculation with *A. piechaudii.* The bacteria did not affect the content of sodium and slightly (but significantly) increased the levels of phosphorous and potassium in the plant tissues. A similar effect was found with both *Pseudomonas fluorescens* strain TDK1 and *P. putida* strain UW4, which contain ACC deaminase (Saravanakumar and Samiyappan, 2007, Cheng et al., 2007). Both wild type strains increased in plant resistance to saline conditions but mutants with lower ACC deaminase activity lost their ability to promote plant growth under salinity stress. *Burkholderia phytofirmans* with ACC deaminase activity increase potato and grapevine resistance to heat stress or ambient temperature stresses (Barka et al., 2006, Bensalim et al., 1998). Strains *Pseudomonas brassicacearum* Am3, and *Pseudomonas* sp. Dp2 with ACC deaminase activity isolated from soil contaminated by heavy metals promoted the growth of rape and pea which were cultivated in cadmium-supplemented soil (Belimov et al., 2001). One explanation for the beneficial effects of PGPR containing ACC deaminase could be suppression of negative effects of ethylene accumulation, and thus help plants cope better with different environment conditions (Ecker, 1995).

In addition to the stresses mentioned above, PGPR with ACC deaminase activity

promoted growth of drought treated plants (Mayak et al., 2004b, Belimov et al., 2009b). *A. piechaudii* ARV8 with ACC deaminase activity significantly increased the biomass of pepper and also improved the growth recovery when watering resumed (Mayak et al., 2004b). *Variovorax paradoxus* 5C-2 containing ACC deaminase activity increased growth of roots and shoots and diminished xylem ACC concentration of pea plants exposed to soil drying, indicating root and shoot transport of ACC is involved in 5C-2 growth promotion on plants (Belimov et al., 2009b). Although ACC deaminase--containing bacteria can regulate ethylene accumulations in young seedlings and stimulate plant growth (Mayak et al., 2004a, Mayak et al., 2004b), details of the long distance signaling pathways involved in these plant-bacteria interactions have not been well described. For example, does the inoculation of rhizobateria such as 5C-2 to plant roots regulate ethylene production of mature leaves? And whether bacteria inoculation could affect the down-stream signaling pathway of ethylene under stress conditions? Could ACC-d containing bacteria be a tool to investigate ethylene function in plant response to stresses? Therefore, some research works here were designed to investigate these questions and further the understanding ACC deaminase containing PGPR and plant signaling interactions.

1.5 Overview of the investigations presented in this thesis

Ethylene has been shown to inhibit floral transition in *Arabidopsis*. As ACC deaminase containing rhizobacteria which can reduce ACC concentrations – ethylene precursor in the root and xylem sap was used in this study to test whether rhizobacterial inoculation on *Arabidopsis* can regulate its floral transition, meanwhile growth. Although ethylene has been suggested as the key element in regulating plant

responses to the inoculation of ACC deaminase containing rhizobacteria by using ACC deaminase mutant strain or ethylene synthesis / action inhibitor of plants, ethylene insensitive or over-producing mutants were used here to further explore ethylene in regulating the interaction between ACC deaminase containing strain *V. paradoxus* 5C-2 and *Arabidopsis*. In second part of this thesis, the growth promotion effect of ACC deaminase containing rhizobacteria was tested in wheat as it is an important crop in UK. Particularly, stomatal response to soil drying was studied by using ACC deaminase containing rhizobateria and ethylene inhibitor 1-MCP as it is suggested ethylene is involved in regulating stomatal response to ABA which is a key hormone in controlling stomato response to soil drying. General methods including growth conditions, ABA and ethylene measurement, and bacterial inoculation were introduced in the chapter 2. Certain methods which were only applied in one chapter were described in that chapter. The last part of this thesis is general discussion, which deals with central themes and places the findings of the investigations into the context of a foundation of work on which to build future investigations

Chapter 2 – General Materials & Methods

2.1 Chapter overview

Two model species *Arabidopsis thaliana,* and wheat (*Triticum aestivum*) were used as plant subjects in this study. *A. thaliana* in the family *Brassicaceae* is widely used in the plant sciences as a model organism. Mutant collections of *A. thaliana* allow scientists to dissect the function of components in many pathways including hormone signalling pathways. To explore rhizobacterial effects on hormone signal regulation, the wild type and ethylene-related mutants of *A. thaliana* were used in this study. Wheat is one of the world's major food crops. Ethylene has been shown to be involved in the regulation of wheat growth and grain filling (Balota et al., 2004, Yang et al., 2006). Here, wheat is used to elucidate the possible regulation by ethylene of the stomatal response to water deficit. General information on plant culture, growth conditions, physiological measurements, biochemical and molecular assessments is provided in this chapter. Information on specific materials and method protocols is included in individual experimental chapters.

2.2 Plant propagation and sowing conditions

2.2.1 *Arabidopsis thaliana*

Different lines of *A. thaliana* including mutants in the ethylene biosynthesis and signalling pathways were used as detailed in chapter 3. In an attempt to avoid any surface contamination from microbes, all seeds were surface sterilized before sowing in the substrates by rinsing with 70% (v/v) ethanol, followed by 95% (v/v) ethanol for

1min. The seeds were first kept at 4 °C for 2 days on wet filter paper (Whatman #1) to increase germination rates before sowing on the growth medium. Seeds were sown in separate pots (diameter 5 cm, height 6 cm, Richard Sankey & Son Ltd, UK) containing a mixture of All Purpose Growth medium (SHL multi-pupose; Sinclair Horticulture, UK), horticultural silver sand (Silvaperl; William Sinclair Horticulture, UK), vermiculite (LBS Hort, UK) at the ratio 3:1:0.5 (v/v/v). The growth medium was sieved and then sterilized by autoclaving (121 °C for 15 minutes). Pots were kept in the controlled environment (CE) room with an average temperature of 23 °C ± 2 °C, and at 230 ± 20 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) during a 16-h photoperiod and kept well-watered throughout. The luminaires (light source) - each containing a single 400 W HQI-BT daylight bulb (Osram powerstar; Osram Ltd., Langley, UK) were set 2 meters above the bench. Eighteen days after germination, nutrient solution (modified Hoagland solution - Table 2.1) was used to fertilize the soil (one time only) by watering the plants until drops of water appeared at the bottom of pots.

When individuals of seed lines were grown to bulk up additional seeds, the components of the Arasystem® which built up a dome cover to contain the seeds were applied from the beginning of reproductive shoot growth (ARACON bases and tubes; Betatech bvba, Gent, Belgium). When siliques became yellow, the stems were cut and then shaken over a plastic gauze to separate the seeds from other plant material. Seeds were kept at room temperature for a short term use and at -20 °C for long-term use.

Table 2.1 Hoagland's solution

Macro-component	Stock Solution	Final concentration
KH_2PO_4	1 M	1 mM
KNO_3	1 M	5 mM
$Ca(NO_3)_2$	1 M	5 mM
Iron (Sprint 138 iron chelate)	15 g/L	22.5 mg/L
$MgSO_4$	1 M	2 mM
Minor-component	g/L	mg/L
H_3BO_3	2.86	2.86
$MnCl_2 \times 4H_2O$	1.81	1.81
$ZnSO_4 \times 7H_2O$	0.22	0.22
$CuSO_4 \times 5H_2O$	0.08	0.08
$H_2MoO_4 \times H_2O$	0.02	0.02

2.2.2 *Triticum aestivum* (wheat)

Wheat seeds were surface-sterilized to avoid any microbial contamination. Sodium hypochlorite solution at 5% (v/v) was used to sterilize seeds for 15 min, followed by 10 washes with sterile water. Next, the seeds were soaked in distilled water for 12 hours to allow imbibition of water. Seeds were carefully moved to wet filter paper (Whatman #1) placed on 90 mm petri-dishes. Seeds were pre-germinated on the filter paper for 2 days. Then seedlings with similar root length were transferred to pots which were filled with a growing substrate comprising a 1:1 (w/w) mixture of a loam-based compost (John Innes No. 2, J. Arthur Bowers, Lincoln, UK) and quartz sand. Elemental composition of this mixture was (mg kg^{-1}): total carbon (C)-22000; total nitrogen (N)-1100, nitrate N-210; available phosphorus (P)-30; available potassium (K)-210; pH 6.0 (Belimov et al., 2009 a). Before transfer of the seedlings, each pot was irrigated with tap water until drops of water appeared from the bottom of pots. The seedlings were gently planted into substrate at a depth of 2 cm. The pots with seedlings were covered with black plastic film for two days at 23 °C ± 2 °C to retain substrate moisture. When shoots extended 1 cm above the soil surface, each

pot was moved to a particular growth environment, depending on the particular experiment. Three growth facilities were used for the wheat experiments, including greenhouse, controlled environment (CE) room and Snijder cabinet. The greenhouse is a naturally lit environment with additional artificial lighting of 200 µmol $m^{-2}s^{-1}$ (Sodium bulb 600 W, Osram powerstar; Osram Ltd., Langley, UK), a 12 h photoperiod and minima/maxima temperatures of 15 °C /26 °C. Environmental conditions set for the CE room were: average temperature of 23 °C ± 2 °C, 230 ± 20 µmol $m^{-2}s^{-1}$ photo-synthetically active radiation and a 12 h photoperiod. Wheat plants used in chapter 4 were grown in the glasshouse and then moved to the CE room 1 week before measurements started. The conditions in the Snijder cabinet were: 15 °C for the dark period and 21 °C for the light period; 250 µmol $m^{-2}s^{-1}$ photo-synthetically active radiation; 40%-50% humidity and an 8 h photoperiod which allowed measurements of leaf length to be taken at the start and end of the photoperiod.

2.3 Rhizobacterial application

2.3.1 Bacterial cultures

Bacteria *Variovorax paradoxus* (5C-2) were originally isolated from the root zone of Indian mustard (*Brassica juncea L. Czern*) variety VIR-3129 cultivated in sewage sludge and mining waste (Belimov et al., 2005). Cultures used in this experiment were obtained from collections obtained from The All-Russia Research Institute for Agricultural Microbiology (ARRIAM), St Petersburg. This strain shows ACC deaminase activity *in vitro* (9.3 ± 0.8 µM *alpha-ketobutyrate* KB $mg^{-1}h^{-1}$) and stimulates root elongation of *B. juncea* (variety VIR3129) seedlings on filter paper culture *in vitro* and plant growth in pot trials.

2.3.2 Bacterial inoculation and enumeration

Three days before plant inoculation, bacterial strains were cultured on solid Bacto-Pseudomonas F (BPF) medium (Belimov et al., 2001) at 28 °C. The composition of BPF medium included (g l^{-1}) peptone 10, casein hydrolysate 10, K_2HPO_4 1.5, $MgSO_4$ 1.5, agar 15. The bacterial solution for plant inoculation was prepared by scraping bacteria from the agar plates into tap water. The liquid suspension culture was diluted with water to yield 10^7 cells ml^{-1} as determined by monitoring the optical density (OD) at 540 nm (Ultrospec 2100 *pro* spectrophotometer Amersham company, UK). Bacterial suspensions were added to the substrates to reach a final concentration of 10^6 cells g^{-1} growing substrate.

To assay the population of bacteria on the roots, plant roots were removed from the substrates by thorough shaking to remove adhering substrate particles. Both main and lateral roots were used for analysis immediately after sampling. Plant samples were homogenized in sterile tap water with a sterile mortar and pestle, the homogenates serially diluted in 10-fold steps, and 50 µl aliquots plated in duplicate on BPF agar supplemented with rifampicin at 20 mg l^{-1}, and Kanamycin at 30 mg l^{-1} (to which 5C-2 shows resistance) and nystatin at 40 mg l^{-1} (to prevent fungal growth). Colony forming units (CFU) were counted by comparing with the morphology of the original strain grown on BPF agar after incubation of plates for 4 days at 28 °C.

2.4 Plant physiology and development

2.4.1 Flowering time and whole plant harvest of *Arabidopsis*

Flowering time of *Arabidopsis* was determined by two methods. (i) Recording the

total number of rosette leaves (excluding cotyledons) plus the number of leaves in the inflorescence on the day when the floral stem was 1cm long. (ii) Recording the growing days since sowing when the floral stem was 1 cm long. Flower number was recorded daily after the first flower opened.

The rosette was harvested at four different time points during the growth period and these were variable depending on the genotype. The fresh weight of the rosette was determined using an electronic balance (Precisa 125A; Precisa Balances, Switzerland), leaf number and leaf area by LI-COR LI-3000A area meter (Model LI-3000A area meter, LI-COR inc, Lincoln, USA). Leaf samples were wrapped in aluminium foil immediately after measurement and placed in liquid N_2 for 10 seconds, and then stored at -80 °C for further analysis.

2.4.2 Leaf elongation rate of wheat

To calculate the leaf elongation rate, the leaf length was measured when the photoperiod started and 30 minutes before the photoperiod finished using a ruler. Leaf length measurements were carried out on Leaf 2 and 3 when they emerged. The growth rate was divided into day and night growth rates since under a constant temperature, dark growth rate of water stressed plants can be higher.

2.4.3 Stomatal conductance of wheat

Stomatal conductance of both abaxial and adaxial leaf surfaces was determined by a diffusion porometer (AP-4, Delta-T Devices Ltd, UK) as described by Wilkinson (2009), and mean was calculated. Before each measurement, the ambient humidity was measured and the cycling range was set in a range in which the ambient value fell. The instrument was allowed to equilibrate with the temperature of the growth room or chamber for 20 min before the measurements started. The porometer was

calibrated using a standard plate for which the water diffusion resistances are known. The standard curves were given by plotting the known resistances versus their corresponding porometer readings. If the standard error of the measurements was less than 10%, the calibration was installed prior to sample measurements. Only fully expanded leaves were chosen to assess stomatal conductance and values were obtained from similar positions on equivalent leaves.

2.5 Plant tissue hormone analysis

2.5.1 ABA assay

ABA concentrations were determined in plant samples by using a radioimmunoassay (RIA). The monoclonal antibody (McAb) used here was kindly provided by Dr Geoff Butcher (Babraham Institute). A competitive RIA using the labeled, DL-*cis*, *trans*-[G-^{3}H] abscisic acid (Amersham plc, UK), and the antibody AFRC MAC 252 was employed. The antibody is specific for (+)-ABA as the free acid and aqueous extracts of wheat leaf tissue which showed no evidence of immunoreactive contamination (Quarrie and Galfre, 1985, Quarrie et al., 1988). Consequently, aqueous extracts were prepared without any purification. The cross-reactivities of this antibody are listed in Table 2.2. The protocol used in this study was developed by Quarrie *et al.* (1988). The details of the different steps in the assay are listed in the following section. The tritium label remaining in the samples was counted in a scintillation counter (Liquid Scintillation Analyser 1600-TR, Packard).

Different concentrations of standard ABA were prepared by using synthetic unlabeled (±)-*cis*, *trans*-ABA (Sigma Let., UK). A standard curve of counts recovered versus unlabeled ABA added was produced with each batch of samples. ABA concentration

from samples was calculated by reference to this standard curve after linearization using the "logit" transformation, where the logit transformation of a variable B is given by

$$\text{Logit B} = In\left[\left(\frac{B-Bmin}{Bmax-Bmin}\right)\Big/\left(1-\frac{B-Bmin}{Bmax-Bmin}\right)\right] \text{ to give a straight line}$$

Correlation coefficients ($r^2 > 0.99$) were considered acceptable. If r^2 values were lower than this standard curve, samples were re-run.

ABA extraction

Plant leaves for ABA determination were kept at -20 °C until required. Tissue samples of around 50 mg fresh weight were freeze-dried in 1.5 ml Eppendorf tubes for 48 h before ABA extraction. Leaf tissue was cut to powder using fine scissors in the tubes. The Eppendorf tube was weighed before any tissue was put in and after the tissues were cut to powder, with the difference considered as the dry weight of the tissues. Milli-Q level water was added to the tube at a ratio of 1:25 (dry weight:water). All tubes with samples were placed on a shaker in a dark cold room (<10 °C) overnight to extract ABA from tissue samples. After shaking, tubes were centrifuged for 5min at 12000 rpm and supernatant was transferred to a new 1.5 ml Eppendorf tube. The supernatant can be stored at -20 °C until required for the radioimmunoassay.

Table 2.2 Specificity of the monoclonal antibody AFRC Mac 62 for (S)-2-cis-abscisic acid (Quarrie et al., 1988, Walker-Simmons et al., 1991). Mac252 showed similar specificity as Mac 62 (Borel et al., 1997)

Compound	Percentage cross-reaction
(S)-2-cis-abscisic acid	100
(S)-2-trans-abscisic acid	0.9
(RS)-2-cis-abscisic acid	51
(S)-2-cis-abscisic acid methyl ester	0.4
(S)-2-cis-abscisic acid glucose ester	0.1
Phaseic acid	0.1
Dihydrophaseic acid	0.1
Xanthoxin	0.1
Abscisic alcohol	0.002
Abscisic aldehyde	0.2

ABA Immunoassay

This method is based on a method previously described by Quarrie *et al.* (1988). 200 µl of 50% (v/v) phosphate buffer saline (PBS) (50 mM Na_2HPO_4 and 100 mM NaCl, adjusted to pH 6.0) was added to each 2 ml tube supported in a foam rack. Fifty µl of sample solution, or standard solution of known ABA concentration (ranging from 0-2000 pg ABA per 50 µl) was added to the PBS. Then 100 µl tritiated ABA dissolved in buffer mixture (5.0 mg/ml globulin dissolved in PBS) and 100 µl MAC 252 dissolved in buffer mixture (5.0 mg/ml bovine serum albumin and 4.0 mg/ml polyvinylpyrrolidone dissolved in PBS) were added. Tubes were capped and mixed by gentle vortexing whilst in the foam rack and placed in the fridge for 45 min, after which samples were centrifuged for 1 min. Saturated ammonium sulphate solution was added to the samples prior to incubation in the dark at room temperature for 30 min to precipitate the ABA-antibody complex. After incubation, samples were centrifuged at 8000 g for 5 min to pellet the precipitate and the supernatant removed. Pellets were washed with 1 ml 50% ammonium sulphate to remove excess unbound radioactivity. Tubes

were centrifuged for 5 min at 8000 g to drain the excess liquid from the sample. To dissolve the pellet, 100 µl dd water was added, followed by vortexing. Scintillant cocktail (1.4 ml - Ecoscint H, National Diagnostic, NJ, USA) was added and then each tube was thoroughly vortexed, individually. After that, each tube was placed in a 20 ml clean glass scintillation vials in a counting rack and samples were counted overnight (6 min per sample) in a scintillation counter (Liquid Scintillation Analyser 1600-TR, Packard). Data were presented as cpm (counts per minute) and the concentration of ABA was calculated from a calibration curve by interpolation.

2.5.2 ACC assay

A gas chromatography-mass spectrometry (GC-MS) method was used to detect ACC extracted from mature leaves. Although a GC method for ACC quantification based on the oxidative conversion of the extracted ACC to ethylene has been widely used in the past thirty years, it shows low accuracy (Coleman and Hodges, 1991). Penrose *et al.* (2001) developed the WatersACCQ-Tag amino acid method to quantify ACC from roots of canola seedlings, but no internal reference can be included in the assay. Furthermore, Penrose et al. (2001) detected that ACC degraded during the time of sample preparation. Although the amount of ACC can be quantified by using an external ACC standard curve, the linearity of standard curve is between 1 and 25 pmol. More effort is then required to determine the ACC range in different samples. The GC-MS method developed by Smets et al (2003) overcomes these weaknesses. Pentafluorobenzyl bromide (PFBBr) used in this method allows sensitive detection of ACC by using GC-MS in negative chemical ionization mode. Therefore, the method used here was based on Smets et al. (2003).

Methanol (80% v/v) was used to extract ACC from plant tissues (100-150 mg) overnight at -20 °C. Fifteen ng [$^2H_4$] ACC was added as an internal standard

(Olchemim, Olomouc, Czech Republic) with methanol. The following procedures were modified from Dodd *et al.* (2009b) and Smets *et al.* (2003). After centrifugation (2000 rpm, 15 min, 4 °C), the supernatant was transferred to a C_{18} cartridge (DSC-18 100 mg, Sigma). The elution was dried to an aqueous phase and the pH was adjusted to 2 by adding 0.01 M HCl. Then the samples were purified by a strong cation-exchange resin (Extract Clean SCX 200 mg/ml, Grace Davison Discovery Sciences, Lokeren, Belgium). After sample loading, the cartridges were rinsed with 3 ml of water: methanol (1:8 v/v). Next, 4 M ammonium hydroxide (750 µl) was used to elute ACC from the cartridge. The eluent was dried by a stream of nitrogen and kept at -20 °C. Samples were dried carefully as any residual moisture in the samples will strongly affect derivatisation in the following steps.

The samples were derivatised by the method described by Smets et al. (2003) and Dodd *et al.* (2009b). Aqueous MeOH (80% v/v) was used to transfer samples to brown-glass vials for derivatisation. The samples were dried under nitrogen, and dissolved in 60 µl acetone. 2 µl of 1-ethylpiperidine and 10 µl of bromopentaflurotoluene were added to the vials and vials were incubated at 60 °C for 45 min. Samples were then purified by liquid-liquid extraction (ethyl acetate-water). The ethyl acetate fraction was dried by nitrogen and then re-suspended in 30 µl methanol for injection onto the GC-MS. In this experiment, ACC-*bis*-pentafluorobenzyl samples were injected by auto-injection onto a gas chromatograph connected to a mass spectrometer (6890N GC and 5975 Inert MSD, Agilent Technologies, UK).

2.5.3 Ethylene assay

In this study, the gas chromatograph (GC) was used to measure ethylene emission from plant leaves (Wilkinson and Davies, 2009). Mature leaves were removed from

plants, immediately weighed, and then placed in glass vials (of different volumes depending on the size of leaves) containing water saturated filter paper. The vials were flushed for 1 min with fresh air from outside the laboratory and then immediately capped with rubber septum lids. Samples were incubated for 60 min under illumination providing 100 µmol PPFD $m^{-2}s^{-1}$. Gas from the vial headspace was extracted (1 ml) with a disposable plastic syringe and manually injected into a gas chromatograph (6890N, Agilent Technologies UK Ltd, Wokingham, UK) fitted with a J&W HP-AL/S (50 m × 0.537 mm × 15.0 mm) column (HiChrom Ltd, Reading, UK). The temperature was maintained at 100 °C for 5 min to resolve ethylene and then increased at 15 °C min^{-1} to 150 °C and held for 1.5 min to remove the water vapour introduced into the column by sample injection. The helium carrier gas was set at a flow rate of 5.7 ml min^{-1} and detection was by flame ionization. Ethylene concentration was calculated with reference to peak areas of known ethylene standards (BOC Special Gases, Manchester, UK) and corrected for tissue fresh weight and the time of incubation to determine ethylene emission rate.

There are some disadvantages in using detached leaves for ethylene quantification because of ethylene production from wounding. Leaves were incubated for only 60 min to minimize the production of wound-induced ethylene (Geballe and Galston, 1982). ACC measurement was included in this study to complement ethylene data.

2.6 RNA manipulation

2.6.1 RNA extraction

Plant tissue samples required for RNA extraction were frozen in liquid nitrogen and stored at -80 °C until extraction. Frozen tissue samples were ground to a fine powder

in liquid nitrogen using a mortar and pestle. In this step, it is important to keep samples frozen by adding additional liquid nitrogen. Total RNA was extracted from approximately 100 mg tissue using the TRIzol reagent from Invitrogen (15596-026), following the manufacturer's instructions. TRIzol reagent contains a mono-phasic solution of phenol and guanidine isothiocyanate and it can separate RNA and DNA into two different phases by working with chloroform, thus avoiding contamination of RNA with DNA. All the material including gloves and plasticware were RNase-free.

The method for using TRIzol reagent includes three major steps: homogenization, phase separation, and RNA precipitation. The ground material of 100mg plant tissues was transferred to 1.5 ml centrifuge tubes. One ml TRIZOL was added to the samples and mixed by vortexing for homogenization. Samples with TRIZOL were incubated at room temperature for 5-10 min to allow the complete dissociation of nucleoprotein complexes. Next, 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added to the supernatant and tubes were vigorously shaken by hand for 15 seconds. After centrifugation in a bench top centrifuge at 7,500 × g for 15 minutes at 2 to 8 °C, the upper aqueous phase which is colorless was transferred to a clean 1.5 ml tube. Isopropyl alcohol was added to the fresh tube with the aqueous phase with a ratio of 0.5 ml per 1 ml of TRIZOL reagent used for the initial homogenization. Then samples were incubated at 15 to 30 °C for 10 minutes and centrifugation was carried out at 12,000 × g for 10 minutes at 2 to 8 °C. After centrifugation, RNA pellets were precipitated on the bottom of tubes. RNA was re-precipitated and re-pelleted by the addition of 1 ml 75% (v/v) cold ethanol, followed by centrifugation in a micro-centrifuge at 7,500 × g for 5 minutes at 2 to 8 °C. Pellets were drained and left to dry on the bench for around 30 min, after which pellets were re-suspended in RNase-free water (500 mg material with 40 µl water) by passing the solution a few times through a pipette tip, and then were incubated for 10 minutes at 55 to 60 °C.

2.6.2 RNA quantification and agarose gel assay

RNA was diluted 25-50 times using RNase-free TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer and quantified using a spectrophotometer (Ultrospec 2100 *pro*, Amersham company, UK) and UV microcuvettes (Life Science Products). Absorbance at 260 nm was recorded to calculate the RNA concentration.

RNA from samples was loaded onto a 1.2% (w/v) agarose gel (Sigma) which was made by heating agarose in a microwave with 1 × TAE buffer (40 mM Tris acetate and 12 mM EDTA, pH 8.0) and then cooled down and allowed to set in a gel casting tray for analysis. Ethidium bromide was added to the agarose gel before it was cooled down. The gel was submerged in an electrophoresis tank (Bio-Rad) filled by 1 × TAE buffer. Purified RNA (1 µl comprising 1-5 µg) was loaded to each well of the agarose gel with RNase-free loading buffer (50% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue in RNase-free water. All chemicals were from Sigma). Electrophoresis was performed at 90 V for 30 min. RNA was visualized by exposure on a UV transilluminator and gel images were obtained using a Biodoc-IT™ Gel Documentation System (UVP).

2.7 Gene expression analysis by Reverse transcription PCR (RT-PCR)

RT-PCR allows semi-quantitative determination of changes in mRNA abundance in parallel samples. It includes two step, cDNA synthesis and cDNA polymerase chain reaction (PCR). RNA (2.5 µg) was used for the first reverse transcription (RT) from each sample and mixed with Oligo dT_{15} (MWG Biotech, Germany) 1 µg. The mixture was kept at 70 °C for 5 min, and then cooled on ice for 2 min. After cooling, M-MLV reverse transcriptase 200U (M170A Promega UK), dNTPs 1.25 µl at 10 mM

concentration (U151A Promega UK) and recombinant RNasin Ribonuclease inhibitor 25U (N2511 Promega UK) were added into the RNA mixture. Samples were briefly centrifuged and incubated at 37 °C for 1 h.

PCR was carried out using a thermocycler (Mastercycler gradient, Eppendorf company, UK) according to the manufacturer's instructions. Subsequently, 2 µl cDNA was used for the polymerase chain reactions (PCR) with pairs of gene sequence-specific primers. Each 25 µl PCR reaction contained 50 ng forward and reverse primer and 20 µl PCR ready mix (1.1* Reddy Mix from ABgene) which includes Taq DNA polymerase and dNTPs. A control gene such as Actin was used to ensure equivalent amounts of cDNA from different samples (Testa et al., 2002) . Amplification proceeded as described in Table 2.3. For each sample and gene of interest, triplicate PCR reactions were performed. 1-1.2% (v/v) agarose gel dissolved in 1 × TAE buffer (described above) was made for electrophoresis. DNA marker 100bp and 1kb (New England BioLabs, UK) was used to determine the size of PCR product.

Table 2.3 PCR parameters

Step	Time	Temperature °C	Number of cycles
Initial denaturing	3 minutes	94	1
Denaturing	45 seconds	94	30
Annealing	45 seconds	50-55	
Extension	1 minute	72	
Final Extension	5 minutes	72	1

Chapter3 – ACC deaminase containing rhizobacteria promote growth and development of *Arabidopsis* via an ethylene-dependent pathway

3.1 Introduction

The triple response of dark-grown seedlings is a classic example illustrating that ethylene inhibits plant growth by reducing hypocotyl elongation and root growth (Guzman and Ecker, 1990). Application of ethylene or its precursor ACC or the ethylene-releasing chemical ethephon (which both can be converted to ethylene by plants) reduced leaf expansion and shoot growth (Lee and Reid, 1997, Pierik et al., 2006). Treatment with ethephon and ACC reduced area of primary leaves of sunflower in a dose-dependent manner (Lee and Reid, 1997). Leaf growth inhibition caused by ACC and ethephon were reversed by pre-treating the plants with silver thiosulphate, an inhibitor of ethylene action. Pierik (2006) summarized exogenous ethylene dose-response relationships in different plant species and tissues. Cucumber root elongation showed a near linear reduction with increasing ethylene concentration. Low levels of ethylene showed a small stimulatory effect on the growth of *Arabidopsis* hypocotyls and wheat coleoptiles, and then with an increase of ethylene concentration, both tissues showed a reduction of growth. Similar response was also observed in slow-growing *Poa alpine* and *Poa compressa* in terms of leaf growth (Fiorani et al., 2002). In addition to plant growth regulation, A delay of floral transition time was observed in *Arabidopsis* by using exogenous ethylene treatment and assessments of *ctr1-1* mutants in which the ethylene pathway is constantly stimulated (Achard et al., 2007). GA treatment restored floral transition which was inhibited in *ctr1-1* mutants or was inhibited in wild type by ethylene. Further evidence

suggested that ethylene regulated plant floral transition via DELLA proteins which belong to a family of nuclear growth repressor proteins mediated pathway.

However, most studies addressed ethylene function in plants by using exogenous ethylene gas, the ethylene-releasing chemical ethephon, or the ethylene precursor ACC to treat plants. Alternatively, ethylene biosynthesis inhibitors or ethylene antagonists or ethylene insensitive mutants were used to investigate ethylene function during plant growth and development. However these approaches only allow elucidation of 'on' and 'off' effects of ethylene rather than dose related effects. In contrast to these studies, only a few studies have looked at regulation of endogenous ethylene and its influence on developmental processes. McDonnell et al. (2009) demonstrated that the *Arabidopsis* genome contains genes which are bacterial ACC deaminase-like. Down-regulating one of the ACC deaminase-like genes in plants caused significantly more ethylene production. Bacterial ACC-deaminase has been transformed into plants such as tomato and canola to control ethylene responsive phenotypes (Biswas et al., 2008, Lopes and Reynolds, 2010). ACC deaminase gene driven by constitutive promoter 35S was expressed in tomato and a significant reduced ethylene production in leaves and fruit was observed in transgenic plants together with significant delays in ripening (Klee et al., 1991). Canola with root over-expressed ACC deaminase showed improved tolerance to nickel stresses (Stearns et al., 2005). Compared to transgenic approaches, soil inoculation of bacteria containing ACC deaminase offers another way to explore ethylene function by regulating endogenous ethylene.

A group of PGPR which contain the enzyme ACC deaminase can decrease the ACC concentration in the root (Penrose et al., 2001) and mitigate ethylene's inhibitory effects on the shoot (Belimov et al., 2009b, Belimov et al., 2005, Glick, 2005, Glick et

al., 1998). Inoculation of ACCd containing bacteria decreased ACC levels in the xylem sap (Belimov et al., 2009 b) and decreased ethylene production of plant roots (Madhaiyan et al., 2006, Mayak et al., 2004a, Mayak et al., 2004b, Penrose et al., 2001). Ethylene biosynthesis or perception inhibitors were used to determine whether the growth promoting effects of bacteria were ethylene mediated and results showed similar effects as bacterial inoculation (Belimov et al., 2002, Belimov et al., 2009 b). An ACC deaminase minus mutants of *Variovorax paradoxus* 5C-2 did not show any distinct growth promoting effects on pea in contrast to the wild-type strain (Belimov et al., 2009 b). Except the usage of ACCd mutant strain, only a few studies have examined the linkage between effects of bacterial growth promotion to ethylene, particularly effects of bacterial inoculation on the ethylene production of mature shoots tissues or on the ethylene signalling pathway of plants. Lopez-Bucio J et al. (2007) used ethylene or auxin signalling defective mutants to investigate effects of PGPR strain *B. megaterium* on auxin or ethylene signalling transductions which could be the explanations of altered root architecture of bacterial inoculated *Arabidopsis*. Following this idea, ethylene insensitive mutants were used here to explore whether *V.paradoxus* 5C-2 mediate plant growth via an ethylene-dependent pathway.

Two ethylene insensitive *Arabidopsis* mutants were used in this study including *etr1-1*, and *ein2-1*. As discussed in chapter1, the stimulated ethylene signal pathway includes EIN2 protein activation and the accumulation of EIN3 or EIN3-like proteins (Roman et al., 1995, An et al., 2010) (Figure 1.2.). Studies on EIN2 protein which mediates the step downstream of CTR1 protein and upstream of EIN3 protein suggests EIN2 encodes a novel integral membrane protein which is similar in its amino acid sequence to the members of a family of disease-related metal-ion transporters like the natural resistance-associated macrophage protein (Nramp) (Alonso et al., 1999,

Roman et al., 1995). EIN3 and the EIN3-like (EIL) proteins belong to a family of transcription factors, which act downstream of EIN2 protein (Roman et al., 1995). *EIN3* gene expression is not induced by ethylene but EIN3 protein is constantly degraded through via the proteasome-mediated degradation pathway if EIN2 protein is not active (Guo and Ecker, 2003, Chao et al., 1997). However, *EIL1* gene expression is negatively regulated by ethylene application (Van Zhong and Burns, 2003, De Paepe et al., 2004). EIN3 and EIN3-like proteins can stimulate the transcription of transcription factor ETHYLENE-RESPONSE-FACTOR1 (*ERF1*), a member of *AP2*-like DNA binding transcription factors family, referred to as ethylene-response-element binding proteins (EREBPs) (Chao et al., 1997, Solano et al., 1998), and then induce certain gene expressions. *EBP* (ethylene-responsive element binding protein) gene encodes a member of the ERF (ethylene response factor) subfamily B-2 and transgenic studies suggests it is downstream of *EIN2*, but not under *EIN3* (Buttner and Singh, 1997). *EBP* can be up-regulated by ethylene application (Buttner and Singh, 1997, Van Zhong and Burns, 2003).

The rhizobacterium *V. paradoxus* strain 5C-2 (Belimov et al., 2005) was used in this study since it contains high levels of ACC deaminase *in vitro*, and can use ACC as its sole nitrogen or carbon source. It stimulates growth of different species under different conditions such as root growth of Indian mustard (*Brassica juncea*), especially under high cadmium conditions (Belimov et al., 2005); root and shoot growth of pea plants in a pot experiment (Belimov et al., 2009 b) and field trial under drought conditions (Teijeiro unpublished data 2009b); and weight of potato tubers of plants growing under drought conditions (Belimov et al., 2009 a). However, the impact of *V. paradoxus* 5C-2 on *Arabidopsis* have not been examined, particularly the effects of bacteria on development.

This study aims to investigate the impacts of *V. paradoxus* 5C-2 on the growth and development of *Arabidopsis* to further understand the signaling pathways in plant and bacterial interactions by exploiting the genetic resources available in *Arabidopsis*. A series of ethylene related mutants were used to explore the ethylene signaling pathway in regulating effects of *V. paradoxus* 5C-2 on growth and development. The ACC concentrations and ethylene accumulation of mature leaves were assessed to determine whether rhizobacterial root inoculation of growing substrate affects long-distance ethylene signalling. Furthermore, expression of ethylene responsive genes was examined to study the down-stream signaling response of ethylene in the mature leaf. Two ethylene responsive genes (*EIL1* and *EBP*) were selected to determine ethylene response under bacterial inoculation since *EIL1* gene expression is negatively regulated by ethylene while *EBP* gene expression is positively regulated by ethylene. It is hypothesized here that *V. paradoxus* 5C-2 stimulated plant growth via an ethylene-dependent mechanism, thus ethylene insensitive mutants should how no growth stimulation in response to inoculation.

3.2 Materials and Methods

Seed lines and bacterial inoculation

Lines used in this study were *Arabidopsis thaliana Columbia* (Col) wild-type, the ethylene insensitive mutants *etr1-1, ein2-1,* and the ethylene over-producing mutant *eto1-1* (Bleecker et al., 1988, Guzman and Ecker, 1990, Roman et al., 1995). All mutant lines were derived from parental *A. thaliana Columbia*. *etr1-1* and *ein2-1* were kindly given by Dr Mike Roberts (Lancaster environment center, Lancaster University, UK) and *eto1-1* mutant was obtained from NASC (European Arabidopsis Stock Centre) stock center. Surface sterilized seeds were kept at 4 °C for 2 days and then sown on top of the growth medium (All Purpose Growth medium- Sinclair Hort

Products, UK- mixed with sand and vermiculite at ratio of 3:1:0.5 v/v/v). Details of growth conditions were given in chapter 2.

Bacterial strains grown on BPF medium were used for liquid suspension preparation. Liquid suspension was applied to the plant growth medium by thoroughly mixing prior to filling the pots. The final bacterial concentration in the growth medium was 10^6 cells g^{-1} compost. *Arabidopsis* seeds were planted (4 seeds per pot) on the surface of the soil. Seventy pots were used for each genotype, with half used as a control, and half irrigated with bacterial suspension. Thirty five plants were kept in a tray with the propagator lid with the vent open. Thirty plants were used to determine the response of flowering and growth, while five plants were used to enumerate bacterial colonization of the root system. Ten days after planting (DAP) the seeds, seedlings were thinned to one seedling per pot.

Physiological and biochemical measurement

To determine the fresh weight of the rosette, leaf number and leaf area, plants were harvested at four points during the growth period: 15 days after planting (DAP), 17 DAP, 21 DAP, 29 DAP for wild type and 33 DAP for *etr1-1* or 35 DAP for *ein2-1*.

To determine ethylene production, mature leaves (around 0.5 g) from wild type plants and the *eto1-1* mutant at a stage 1 or 2 days before bolting were taken and placed in 7.8 ml glass vials to incubate for 1 hour. Leaf samples for ACC measurement were also collected 1 or 2 days before bolting.

Analysis of gene expression by reverse transcription PCR

Rosette leaves of *Arabidopsis* which are 1 or 2 days before bolting were collected and frozen in liquid nitrogen. Samples were stored at -80 °C until analysis.

Primers used in RT-PCR

EIL1 (AT2G27050):

Forward 5'-CGGCGAAAGAGAGTGCTACTT-3'

Reverse 5'-TCCTTCCATTGCTCCGGTTTG-3'

Size of DNA: 851bp

EBP (AT3G16770):

Forward 5'-TTATTTCCGATTATGCC-3'

Reverse 5'-CGTACCAAGCCAAACTCTAAC-3'

Size of DNA: 543bp

Actin2 (AT5G09810):

Forward 5'- GGCCGATGGTGAGGATATTC-3'

Reverse 5'- CCGCAAGATCAAGACGAAGGA-3'

Size of DNA: 548bp

Statistics

Pairwise comparisons used Student's t-tests and standard error (SE) in SigmaPlot for Windows Version 7.0 (Jandel Scientific, Erkrath, Germany). Two way analysis of variance (ANOVA) was performed to determine effects of rhizobacteria, genotype and their interactions with SPSS version 19 (SPSS Inc, Chicago, USA).

3.3 Results

Flowering time of *Arabidopsis* was assessed by determining the number of growing days since sowing and the rosette leaf number when the plant stem extended to 1cm. Wild type plants (*Columbia* genotype) inoculated with *V. paradoxus* 5C-2 flowered

significantly earlier than control plants ($P<0.01$; Figure 3.1). However, ethylene insensitive mutants *etr1-1*, and *ein2-1* which were inoculated with *V. paradoxus* only showed a small but not statistically significant ($P>0.1$; Figure 3.1) increase in flowering days. Inoculation with *V. paradoxus* significantly ($P<0.01$) decreased the number of rosette leaves at flowering (Figure 3.2) but this effect was not found in the ethylene insensitive mutants *etr1-1* and *ein2-1* ($P>0.1$; Figure 3.2). However, effects of *V. paradoxus* inoculation on cauline leaf number of wild type and ethylene insensitive mutants were not significant ($P<0.1$; Figure 3.2).

Inoculation of *V. paradoxus* significantly ($P<0.01$) increased fresh biomass of wild type plants throughout development (Figure 3.3). The total fresh biomass (% of control) in different genotypes which were inoculated with or without *V. paradoxus* 5C-2 was analyzed by means of a two-way ANOVA. The effects of bacterial inoculation and genotype were significant (both $P<0.001$), as was the interaction ($P<0.001$; Figure 3.4). The significant interaction term indicates that bacterial inoculation significantly ($P<0.01$, T-test) stimulated growth of wild type plant but not *etr1-1* and *ein2-1* mutants (Figure 3.4). Similarly, bacterial inoculation and genotype significantly affected leaf area ($P<0.05$ and $P<0.001$ respectively) and, there was a significant interaction between genotypes and bacterial inoculation ($P<0.001$; Figure 3.5). Again, leaf area of wild type plants was significantly ($P<0.01$) increased by inoculation of *V. paradoxus* but there was no promotion effect on *etr1-1* and *ein2-1* mutants (Figure 3.5).

V. paradoxus inoculation also significantly ($P<0.01$; Figure 3.6) increased growth of the ethylene over-producing mutant *eto1-1*. At the end of the experiment, the introduced strain 5C-2 was detected on roots of both wild type plants and ethylene mutant *etr1-1* and *ein2-1* (Figure 3.7), but there was no significant genotypic effect.

However, as not enough root tissues were obtained to analysis bacterial numbers. Bacterial numbers were not determined in the *eto1-1* plants.

The inoculation with the 5C-2 strain ($P<0.05$) significantly decreased ACC concentrations (Figure 3.8) in rosette leaves of mature wild type plants. Furthermore, bacterial inoculation significantly decreased ($P<0.01$) ethylene emission from the rosette leaves of mature wild type and *eto1-1* mutants. Ethylene emission of both genotypes responded similarly to inoculation, as indicated by the showed significant effects on ethylene emission ($P<0.01$), while the interaction of genotype × inoculation is non-significant ($P>0.05$; Figure 3.9).

ACTIN2 (AT5G09810) was selected as a baseline control in all subsequent RT-PCR assays. In this experiment, ethylene response genes *EBP* and *EIL1* were selected to test the molecular response of *Arabidopsis* to bacterial inoculation. Fully expanded leaves from four independent experiments were sampled for gene expression and only two of these showed different expression patterns between bacterial treatment and control. In these batches, bacterial inoculation down-regulated *EBP* expression in wild type and *eto1-1* plants but up-regulated *EIL1* expression in rosette leaves of wild type plants (Figure 3.10).

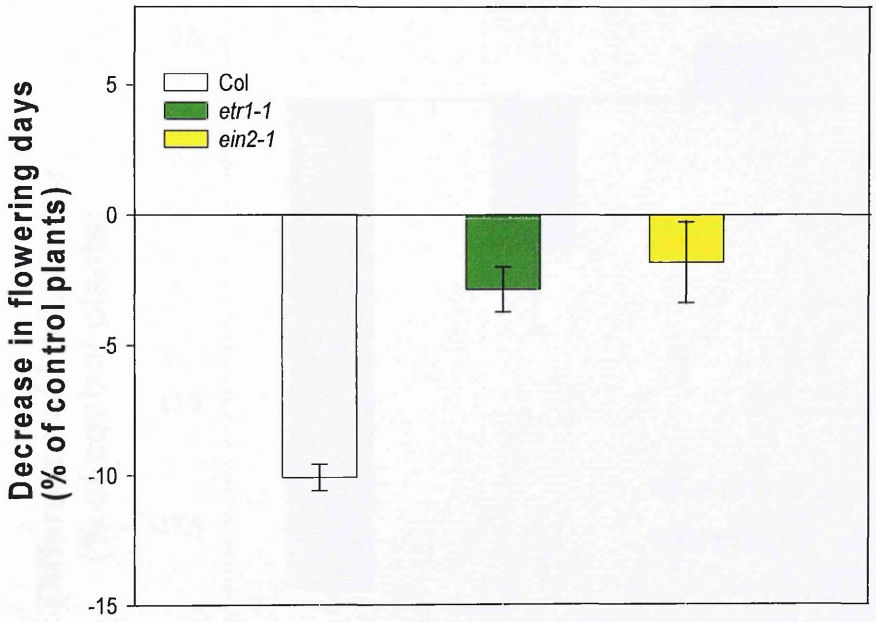

Figure 3.1. Percentage decrease in flowering date of *V. paradoxus* 5C-2 treated wild type, *etr1-1* and *ein2-1* plants. Control plants are 0 compared to inoculated plants. The flowering time was recorded in days after sowing when the floral stem was extended to 1cm. Bars were indicated as mean ± standard error (SE) (n = 30).

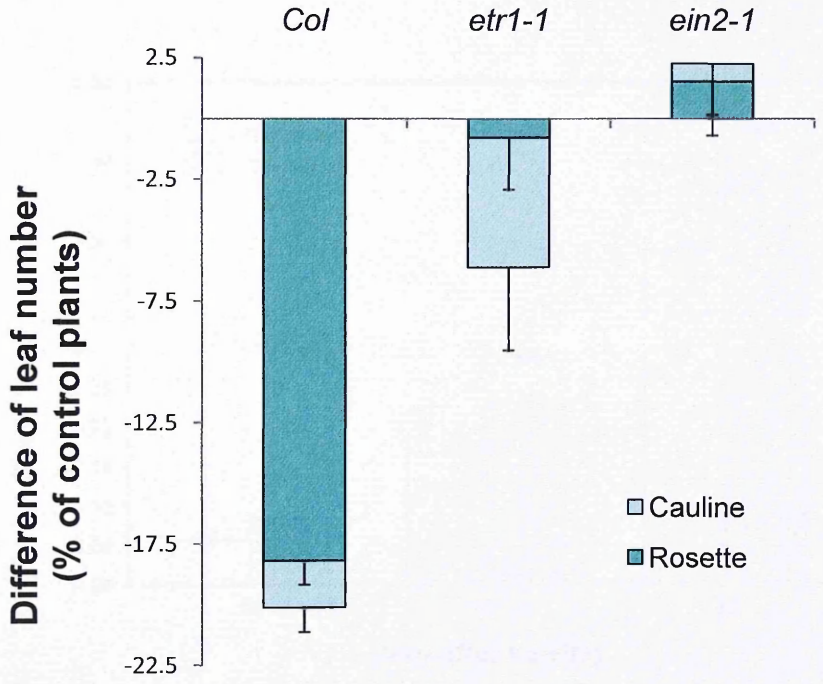

Figure 3.2. The percentage increase or decrease in the leaf number (rosette or cauline leaf) from the *V. paradoxus* 5C-2 treated wild type, *etr1-1* and *ein2-1* plants. Control plants are 0 compared to inoculated plants. Leaf numbers were recorded when floral stem was extended to 1cm. Bars indicates as mean ± SE (n = 30).

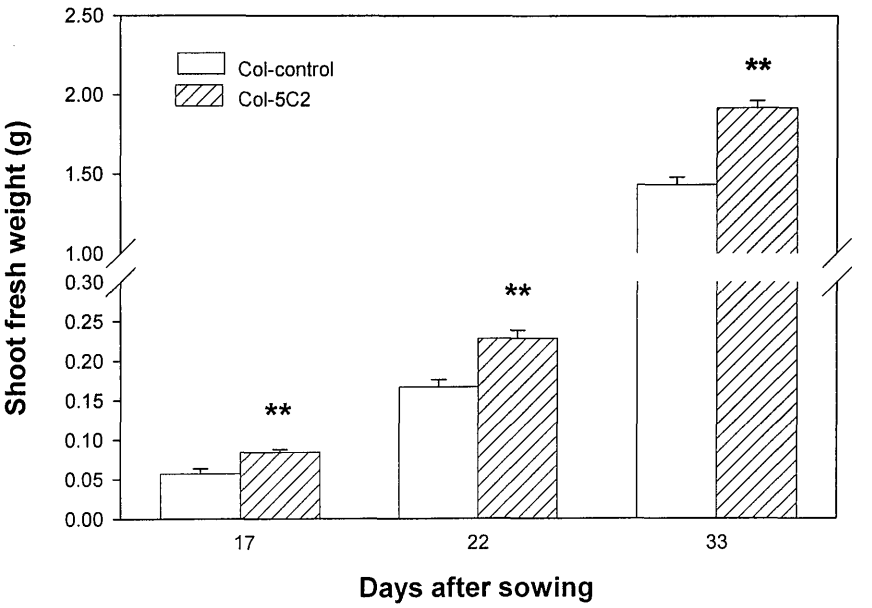

Figure3.3. Fresh biomass accumulation of wild type plants which were harvested at 17 days, 22 days, and 33 days after planting in response to *V. paradoxus* 5C-2 inoculation. Bars indicates as mean ± SE (n=25-30). Asterisks indicate significant difference at $P<0.01$.

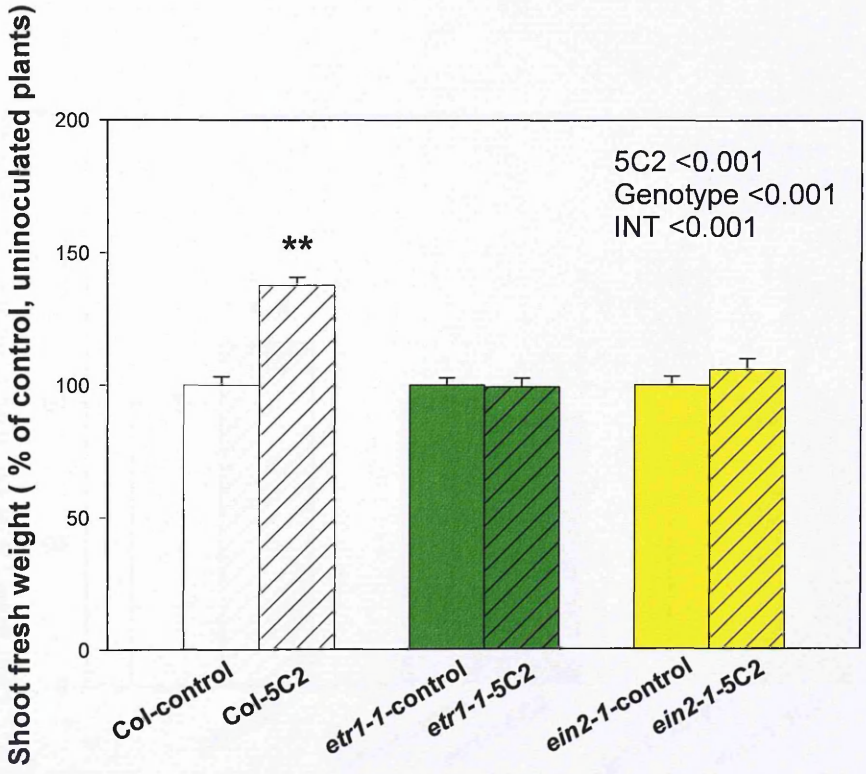

Figure 3.4. Fresh biomass accumulation (% of control plants) of wild type plants and ethylene mutants (*etr1-1*, *ein2-1*) in response to *V. paradoxus* 5C-2 inoculation. Wild type plants were harvested at 29 DAP, while *etr1-1* and *ein2-1* were harvested at corresponding development stage - 33 DAP and 35 DAP respectively. Bars indicates as mean ± SE. Asterisks indicate significant pair wise difference between treatments within lines (** P<0.01). P values are shown for two-way ANOVA for bacterial treatment (5C-2), genotypes (Genotype) and interaction (int).

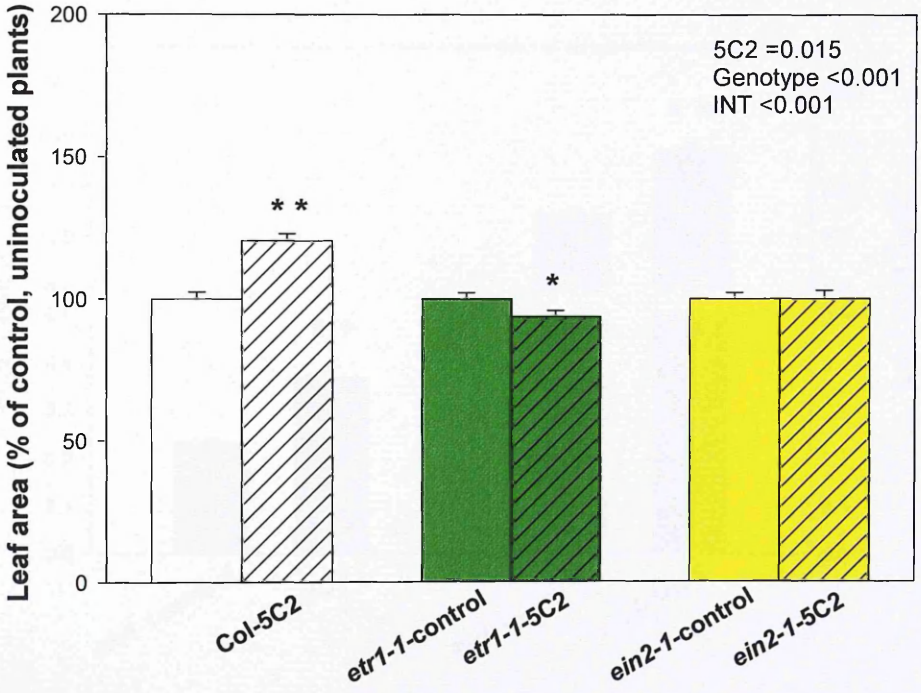

Figure 3.5. Leaf area (% of control plants) of wild type and ethylene mutants (*etr1-1*, *ein2-1*) in response to *V. paradoxus* 5C-2 inoculation. Wild type plants were harvested at 29 DAP, while etr1-1 and ein2-1 were harvested at the corresponding development stage - 33 DAP and 35 DAP respectively. Bars indicates as mean ± SE. Asterisks indicate significant pair-wise difference between treatments within lines (* P<0.05, ** P<0.01). P values are shown for two-way ANOVA for bacterial treatment (5C-2), genotypes (Genotype) and interaction (int).

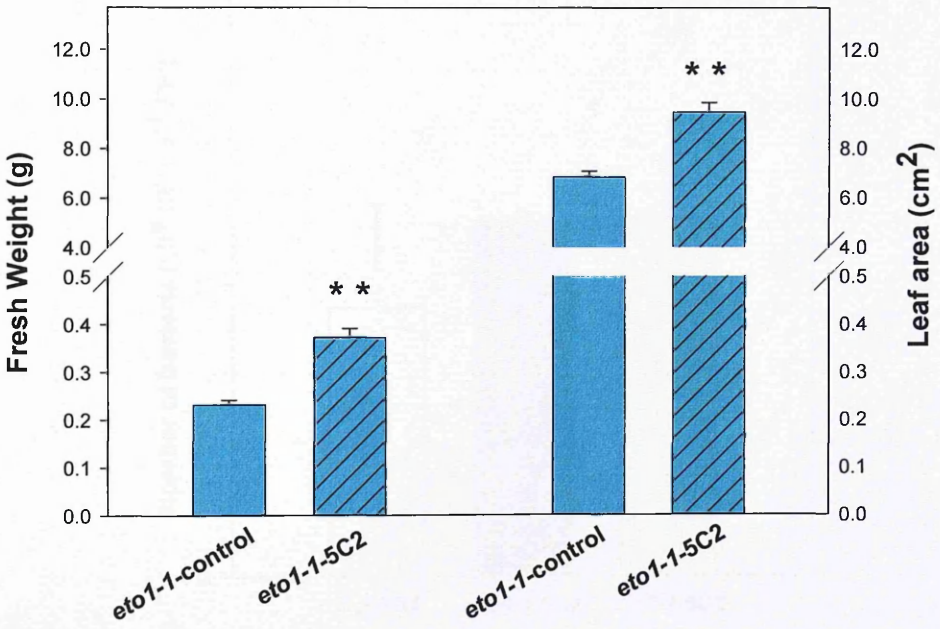

Figure 3.6. Leaf area (2 left columns) and fresh biomass (2 right columns) accumulation of ethylene mutant *eto1-1* in response to 5C-2 inoculation. *eto1-1* was harvested at 28 DAP. Bars indicates as mean ± SE (n=25-30). Asterisks indicate significant difference between treatments ($P<0.01$)

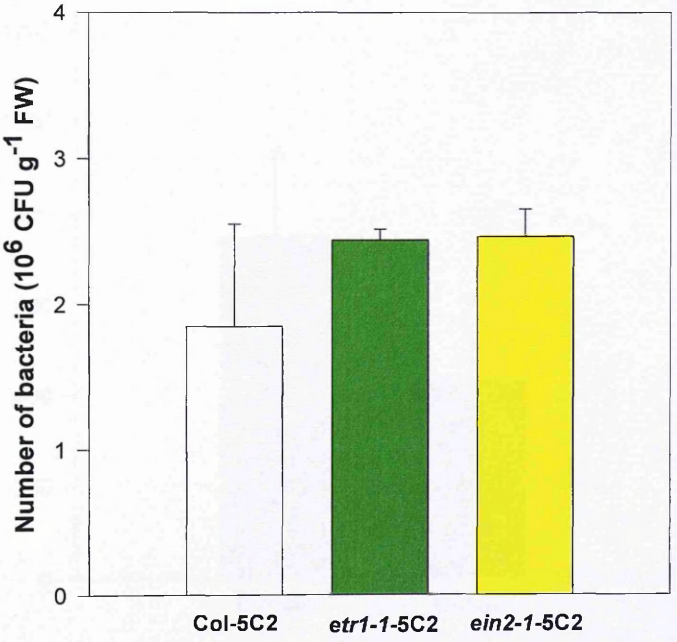

Figure 3.7. The number of bacteria isolated from the root of inoculated wild type, *etr1-1* and *ein2-1* plants. Plant roots were harvested after above-ground tissues were harvest to detect the number of bacteria *V. paradoxus* 5C-2. Bars indicates as mean ± SE (n=4). One way ANOVA showed no effect of genotype on root colonization.

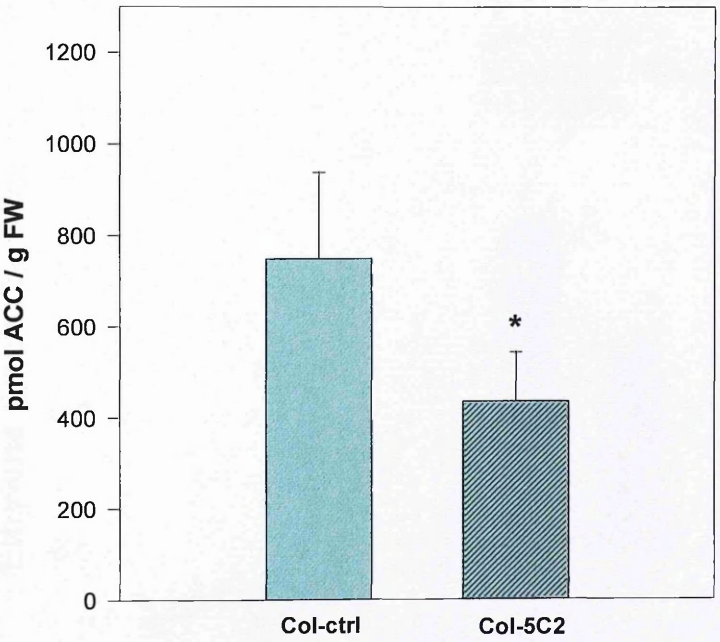

Figure 3.8. ACC concentration in fully expanded leaves of plants (*Col-O*) inoculated with *V. paradoxus* 5C-2 or not. Plant leaves were harvested 1 or 2 days before bolting. Bars indicate as mean ± SE (n=8-10). Asterisks indicate significant difference ($P<0.05$)

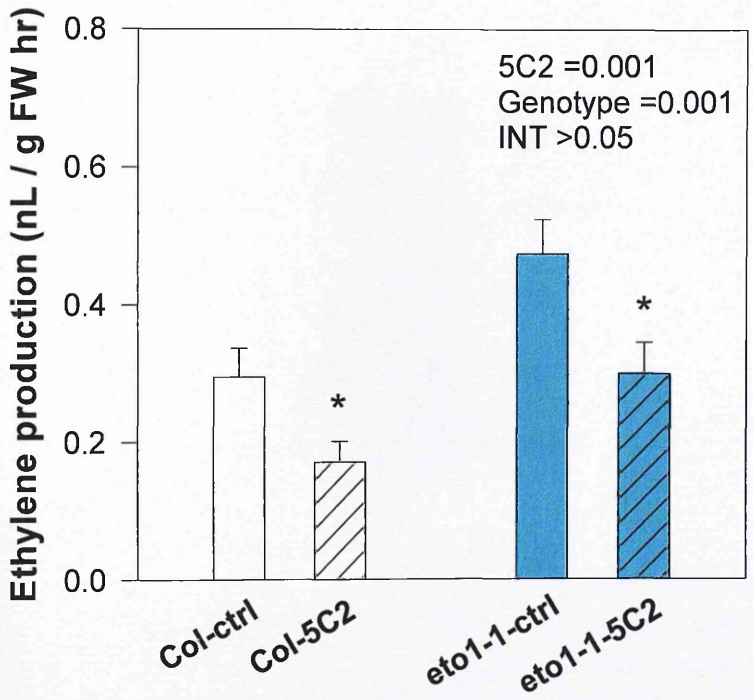

Figure 3.9. Ethylene emission from fully expanded leaves of plants (*Col-O* and *eto1-1*) inoculated with *V. paradoxus* 5C-2 or not. Plant leaves were harvested 1 or 2 days before bolting. Bars indicates as mean ± SE (n=10-12). Asterisks indicate significant difference (P<0.05) between rhizobacterial treatments. P values are shown for two-way ANOVA for bacterial treatment (5C-2), genotypes (Genotype) and interaction (int).

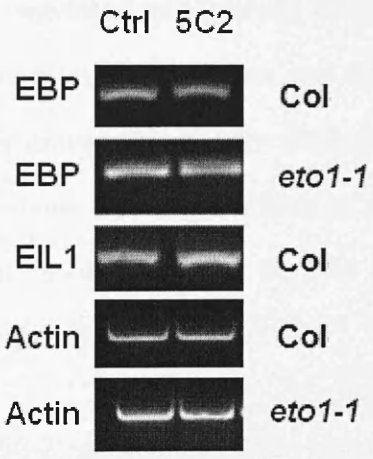

Figure 3.10. RT-PCR shows expressions of ethylene response gene. Expressions of *EBP* and *EIL1* by RT-PCR in control (Ctrl) and bacteria 5C-2 treated plants. Leaf samples of wild type and *eto1-1* mutants were tested. Size of DNA bands: *EIL* 851bp, *EBP* 543bp, and *ACTIN* 548bp.

3.4 Discussion

Plant growth promoting rhizobacteria can trigger developmental changes in the host plant via multiple mechanisms as discussed in the introduction. The responses of plants to PGPRs usually involve several hormone or hormone-related signalling pathways (de Salamone et al., 2001). The work presented here showed that *Variovorax paradoxus* 5C-2 regulated endogenous ethylene production from plants, thereby influencing plant biomass accumulation and the development of the plant. Although this strain also produces IAA *in vitro* (Belimov et al., 2005), the lack of growth stimulation of ethylene insensitive mutants suggested that a functional ethylene signalling pathway is necessary for 5C-2 to stimulate *Arabidopsis* shoot growth and development (Figure 3.1-3.5).

Inoculation of bacteria 5C-2 containing ACC deaminase decreased ethylene production of wild type plants and in the ethylene over-producing mutant *eto1-1* by regulating shoot ACC levels. Meanwhile, stimulation of plant growth and flowering were observed in the inoculated wild type plants. Root inoculation with *V. paradoxus* 5C-2 allowed earlier flowering in wild type plants, but not in ethylene insensitive mutants (Figure 3.1). Furthermore, inoculation of *V. paradoxus* 5C-2 decreased leaf number at flowering compared to control plants (Figure 3.2), indicating 5C-2 promotes *Arabidopsis* flowering by promoting floral initiation. These data support suggestion that exogenous ethylene application or a constantly active ethylene signalling pathway inhibited floral transition (Achard et al., 2003). The growth promotion effect of *V. paradoxus* 5C-2 was observed from 17 DAP to 33 DAP (Figure 3.3), suggesting this effect continued throughout the vegetative growth stages. The promotion of leaf area development and fresh biomass accumulation of wild type plants indicates that *V. paradoxus* 5C-2 induced stimulation of leaf area growth

greatly contributed to the increase of fresh biomass. Two-way ANOVA analysis suggested growth promotion effect of bacterial inoculation was genotype dependent (Figure 3.4, 3.5). Growth promotion of wild type but not ethylene insensitive mutants in response to *V. paradoxus* 5C-2 inoculation indicates that a functional ethylene signalling pathway is necessary for 5C-2 to promote growth of *Arabidopsis*. However, small reduction of leaf area (6.3%; $P<0.05$) was observed in bacterial inoculated *etr1-1* mutants, indicating that bacterial inoculation negatively affect *etr1-1* leaf expansion.

eto1-1 is an ethylene over producing mutant as a result of an increasing in the stability of ACC synthesis protein 5 (Chae et al., 2003). The dwarf phenotype of *eto1-1* is consistent with the idea that high levels of ethylene inhibit plant growth. *V. paradoxus* 5C-2 inoculation partly reversed the *eto1-1* phenotype, both in leaf area and fresh biomass, indicating that decreased ethylene levels can be the explanation for the growth promotion stimulated by *V. paradoxus* 5C-2.

As discussed earlier, there is a lack of studies to show the clear evidence that bacterial inoculation of the soil affects ethylene evolution from shoots of mature plants growing in soil. Although Belimov et al. (2009b) demonstrated that inoculation of *V. paradoxus* 5C-2 decreased ACC levels in xylem sap, which indicates that *V. paradoxus* 5C-2 affect plant signaling systemically, this is still based on the assumption that root ACC export quantitatively contributes to shoot ethylene evolution (Else and Jackson, 1998). However, ACC synthase is encoded by multi-gene families which can be expressed in roots and shoots in different plant species (Wang et al., 2002, Liang et al., 1992, Johnson and Ecker, 1998), indicating that plants can regulate its ACC pool in many ways. These observations raise the question: how does ethylene evolution of shoots and roots respond to bacterial inoculation and is root to

shoot long distance ACC signalling involved in this interaction between *V. paradoxus* 5C-2 and *Arabidopsis*? Results of studies reported here demonstrate that inoculation of bacteria 5C-2 decreased foliar ACC levels and ethylene accumulation in the shoots of mature plants. This suggests bacterial inoculation not only causes local effects on roots but also systemic effects via long-distance ACC signalling.

Decrease in ethylene evolution of mature leaves in response to bacterial inoculation was also found in the *eto1-1* mutant, consistent with the response of leaf area and fresh biomass. The concentration of ethylene from mature leaves of the *eto1-1* mutant was around 2 times higher than from wild type leaves, as previously suggested (Guzman and Ecker, 1990). Absolute values of ethylene emission from mature leaves of both wild type and *eto1-1* (Figure 3.9) differ from those previously reported (Guzman and Ecker, 1990). The difference may be due to the methodological difference such as incubation time of leaves. Higher ethylene emission may have occurred by high CO_2 accumulation since ethylene and CO_2 accumulation can positively feedback and regulate ethylene production (Dhawan et al., 1981, Nakatsuka et al., 1998). Long term incubation of detached leaves may cause wound induced ethylene production (Geballe and Galston, 1982, Coleman and Hodges, 1987). Usually after one hour of wounding treatment, ethylene production starts to increase. While *V. paradoxus* 5C-2 inoculation caused ethylene production from *eto1-1* to decrease to the levels found in wild type plants, the leaf area and fresh biomass were still much less than wild type plants. *eto1* mutant gains a recessive mutation in ETO1 which acts as a substrate adaptor to ACS5 and regulates ACS5 protein degradation (Wang et al., 2004). But ETO1 contains a BTB (Broad-complex, Tramtrack, Bric- a'-brac) motif which offers sites for ETO to interact with other proteins and make ETO muti-functional (Collins et al., 2001). Therefore, inoculation of *V. paradoxus* 5C-2 partly reversed effects of ethylene over production

on growth, but not all of the phenotypes gained from mutation in ETO1.

In previous studies, root inoculation of PGPR *Pseudomonas-thivervalensis* MLG45 which can improve plant resistance to pathogen regulated gene expressions both in roots and shoots (Cartieaux et al., 2003). Meanwhile, incubation of canola roots with *Enterobacter cloacae* UW4 containing ACC deaminase modified the expression of several genes including an RNA binding protein, a cell division cycle protein and defense related proteins (Hontzeas et al., 2004). No ethylene related genes were identified but this may be due to the limited information on the gene sequences in canola. Here it is found that inoculation of bacteria 5C-2 regulated expression of two ethylene response genes in wild type plants in two independent experiments. *EBP* (At3G16770) which encodes an ethylene response factor is induced by ethylene treatment (Van Zhong and Burns, 2003), while *EIL1* (AT2G27050) is one member of the family of ethylene insensitive 3 (*EIN3*) like genes and it is negatively regulated by ethylene treatment (Chao et al., 1997, De Paepe et al., 2004). Although it is shown that bacterial inoculation decreased shoot ethylene emission (Figure 3.9), it is still not clear whether this decrease could affect intracellular signalling. In this study, *EBP* was down-regulated and *EIL1* was up-regulated by bacterial inoculation, indicating bacterial inoculation regulated plant signalling both at the physiological and the molecular levels. However, as results of four experiments showed, inoculation of bacteria regulated the expression of ethylene response genes in only two experiments. Bacterial effects on plant biomass accumulation are statistically significant but also long term effects. RT-PCR only can pick up momentary differences in gene expression at the time of tissue harvest. It is half quantity technique and may not be sensitive enough to pick up differences of gene expressions. How bacteria regulate ethylene signal transduction and how ethylene is involved in hormone net-works requires further investigation. To fully elucidate this response, better

techniques such as real time PCR need to be carried out in different independent experiments to understand how bacterial inoculation affect plant molecular responses.

The work presented in this chapter elucidated that root inoculation of rhizobacteria containing ACC deaminase promoted plant shoot growth and development via ethylene dependent signal pathway by regulating shoot ethylene production. However, the work here only explored bacterial effect on ethylene signaling under optimal conditions. Future experiments should address the regulation of plant signaling net-works under certain stress conditions following bacterial inoculation.

Chapter4 – The response of leaf elongation of wheat to rhizobacterial inoculation in well watered and drying soil

4.1 Introduction

As the soil dries, plant transpiration decreases and leaf elongation slows, which may be interpreted as a water saving mechanism. Both hydraulic and chemical signals are involved in plant responses to drying soil (Bacon et al., 1998, Christmann et al., 2007, Tardieu and Simonneau, 1998, Wilkinson et al., 1998). Water present in the vacuole generates turgor pressure which maintains cell or plant structural integrity. The maintenance of water potential gradients from xylem to expanding cells is required for continued cell expansion. When cell turgor exceeds a threshold value, it generates a demand for water to which allows water to enter by relaxation of the cell walls, thus causing cell water extension (Boyer, 1985, Tomos, 1985). Soil drying can cause a collapse of water potential gradients, thus leading to reduction of cell turgor, and these changes limit leaf growth (Boyer, 1968, Stearns et al., 2005). In addition to hydraulic signals, chemical signals or metabolics also are involved in regulating leaf expansion during soil water deficit (Bacon et al., 1998, Lopez-Bucio et al., 2007, Michelena and Boyer, 1982, Ben Haj Salah and Tardieu, 1997, Wilkinson et al., 1998). The importance of chemical signalling was demonstrated by experiments that maintained leaf turgor as soil dried by applying a pneumatic pressure to roots, leaf elongation can still be inhibited (Pantin et al., 2011, Passioura, 1988). Abscisic acid (ABA) is one of chemical signals which is involved in plant response to soil drying (Davies and Zhang, 1991, Jackson, 1997, Wilkinson and Davies, 2010). It was observed in many species that soil drying can increase ABA accumulation. ABA is transported to the shoot in the xylem sap (Cutler and Krochko, 1999, Davies and Zhang, 1991, Dodd

et al., 2008, Gowing et al., 1993, Wilkinson and Davies, 2002). Once ABA arrives at the guard cell, it can induce stomatal closure to help plants save water (Davies and Zhang, 1991, Loveys, 1984, Wilkinson and Davies, 2002). In addition to stomatal regulation, Sharp and his colleagues (LeNoble et al., 2004, Sharp et al., 2000, Spollen et al., 2000) suggested that ABA functions to maintain, rather than inhibit shoot or root growth at low water potential by restricting ethylene production. Dodd et al. (2009b) also showed that increased supply of ABA from a wild-type rootstock could phenotypically revert an ABA-deficient scion by decreasing xylem ACC concentration and foliar ethylene production.

While it is well recognized that water deficit increases ABA production, there is still some debates about whether water deficit promotes ethylene synthesis as discussed in chapter1. Although now it is not clear that whether ethylene is involved in regulation of leaf growth during soil drying, Belimov et al. (2009 b) showed that rhizobacteria *Variovorax paradoxus* 5C-2 containing ACC deaminase partially restored pea growth which was inhibited by water deficit. Inoculation of plants with rhizobacteria 5C-2 increased root and shoot dry weight of pea grown in the water deficit conditions, but such growth promotion effect was not observed with a mutant M4 of *Variovorax paradoxus* 5C-2 which had lower ACC deaminase activity. Inoculation with rhizobacteria 5C-2 also promoted vegetative growth, tuber yield in potato plants grown in drying soil (Belimov et al., 2009 a). Furthermore, the promotion effect of 5C-2 was also observed in maize plants which subjected to water deficit (Dodd et al., 2009a). Particularly this report suggested that there is no effect of 5C-2 inoculation on leaf xylem ABA concentration. However, ethylene emission from leaves was not examined. In the work described here, possible effects of 5C-2 inoculation were examined in wheat grown in well watered and soil drying conditions. Furthermore, earlier reports suggested elevated carbon dioxide concentrations

increased ethylene emission (Dhawan et al., 1981). Light also stimulated ethylene emission in leaves of *Gomphrena globosa L.*, (Grodzinski et al., 1982, Grodzinski et al., 1983). In sunflower seedlings, two peaks of ethylene emission were observed in a 24 hour period (Finlayson et al., 1991). As light and carbon dioxide vary due to photoperiod and thus could affect ethylene emission, the ACC pool in leaves and roots could be affected by the progress of the photoperiod. It is not clear whether the growth promotion effect of 5C-2 will differ between the light period and the dark period. Therefore, leaf growth rates (LER) in the light period and the dark period were studied to explore whether photoperiod could affect plant responsiveness to bacteria. Attempts were made to measure foliar ethylene emission to explore the potential effect of bacteria 5C-2 inoculation on ethylene production in wheat plants subjected to soil drying.

4.2 Methods

Spring wheat (*Triticum aestivum*) cultivar Ashby was used in this study. Seeds were pre-germinated as described in chapter 2 before they were transferred to pots (L 6cm × W 6cm × H 8cm) filled with a mixture of loam-based compost (John Innes No. 2, J. Arthur Bowers, Lincoln, UK) and quartz sand at a ratio of 2:1. Bacterial strain grown from BPF medium was used to prepare liquid suspensions which were applied to the compost mixture by thoroughly mixing the compost. The final bacterial concentration in the growth compost is 10^6 cells g^{-1} compost. Pre-germinated seeds were planted carefully into the pots with growing substrate. Plants were grown on in a Snijder cabinet under conditions described in chapter 2. All plants were fully watered before applying a soil drying treatment. Two types of soil drying were applied in this study. In experiment 1, plants were kept well watered for 8 days after transplanting and then

water was withheld from plants in the soil drying treatment. Well watered plants were watered every two days to maintain matric potential around -25 –hPa to -55 -hPa. Once leaf number 2 or 3 appeared, leaf length was measured by ruler at the start and end of the photoperiod, until the leaf stopped growing. At the end of the photoperiod, pot weight was measured by placing pots on an analytical balance at regular time intervals to estimate evapotranspiration (ET). Soil evaporation was estimated by using blank pots (without a plant). For experiment 2, plants were kept well watered for 7 days and then soil drying was imposed by supplying plants daily with 50% of the water that was lost through evapo-transpiration (Eta) in the previous 24 hours. 100% of water lost by Eta was delivered to well watered plants daily. In both experiment 1 and 2, four treatments were included: (± soil drying) × (± rhizobacteria).

At the end of each experiment, the introduced strain, 5C-2, was isolated from roots of 3 pots (randomly selected) per treatment (Figure 4.1). After bacterial isolation, growing substrate without above-ground parts from an individual pot was weighed and then transferred to a drying oven (48 hours at 80 °C), then removed and weighed. To determine substrate moisture release characteristics, a calibration curve described by Martin-Vertedor and Dodd (2011), was developed to convert substrate water content to substrate matric potential (Figure 4.2).

Ethylene emission

To study ethylene emission in different treatments, leaf tissues (0.5-1g) from four treatments (well watered or soil drying plants with or without 5C-2) were incubated in 7.8 ml vials to collect ethylene for ethylene concentration quantification by GC method described in chapter.

Statistics

Pairwise comparisons used Student's t-tests and standard errors (SE) in SigmaPlot for Windows Version 7.0 (Jandel Scientific, Erkrath, Germany).

4.3 Results

Experiment 1: *Leaf length Leaf elongation rate*

Soil drying showed an effect on soil matric potential 5 days after water withholding. The matric potential of soil subjected to a drying treatment in both control and bacterial inoculated plants continued to fall until 7 days after water withholding (Figure 4.3). Due to the limit of measurement with a water-filled tensiometer which can not sense the difference in matric water potential when substrate water content is lower than 0.1 g g^{-1}, no difference was found in matric water potential from 7 days but substrate water content continued to decrease, indicating matric potential continued to decrease (Figure 4.4). Soil drying did not show any significant effect on the leaf length of the second leaf or leaf elongation rate (LER) (Figure 4.5) comparing to well-watered control plants. No significant difference of second leaf growth or LER was observed in rhizobacteria treated plants either in well watered or soil drying treatment (Figure 4.6). The second leaf kept growing until 2 days after the third leaf emergence. For the third leaf, a significant decrease in the LER (Figure 4.7, table 4.2) and leaf length (Figure 4.8, table 4.1) caused by the soil drying treatment was first observed from both control and 5C-2 inoculated plants 7 and 9 days after watering was withheld, respectively. Meanwhile, the LER of well watered plants either control or bacterial inoculated reached maximal rate in the light period of 7 days after soil drying started accompanied with reduced rate during dark period, and maintained for

4 days during light period (Figure 4.7, table 4.1). However, the LER of soil drying plants continued to fall and no difference was found between light and dark period either in control or bacterial inoculated plants (Figure 4.7). The third leaf of un-watered plants stopped growing from 9 days after water withholding while the leaf length of well watered plants still increased for 12 days after water withholding (Figure 4.8, table 4.1). However, there is no difference detected in the leaf length and the LER between control and 5C-2 inoculated plants either in the watered treatment or in the soil drying treatment during light or dark period. Colonies of introduced strain 5C-2 were detected on inoculated roots but not from control roots (Figure 4.1). No significant difference was found in bacterial inoculation between watered and those subjected to soil drying.

Experiment 2: *Leaf length and leaf elongation rate*

In experiment 2, soil drying was imposed by supplying only 50% of plant evapo-transpiration, Matric potential showed a gradually decrease 7 days after the drying cycle started (Figure 4.9). The matric potential of drying soil continued to fall but still did not reach -800 –hPa at the end of experiment as shown in experiment 1. During drying cycles, the second leaf continued to grow until 8 days after the drying cycle started and then growth remained at a near steady rate (Figure 4.10). Soil drying did not cause any reduction in the final leaf length and the LER of the second leaf of either well watered plants or plants in drying soil (Figures 4.10 and 4.11). The LER of the second leaf reached maximal rate in the light period of 5 days after drying started and remained steady in the light period for 2 days with a reduction in growth rate in the dark period (Figure 4.11). However, in the third leaf, a decrease caused by soil drying treatments in the final leaf length (Figure 4.12, table 4.2) and the LER (Figure 4.13, table 4.2) was observed at 10 and 11 days after the drying cycle started,

respectively. The LER of the third leaf of 5C-2 and control plants reached maximal rate in the light period of 5 days or 7 days after drying started respectively with well watered. In soil drying plants, the LER reached maximal rate in the light period of 7 or 8 days after drying started in 5C-2 inoculated or control treatments respectively. The LER of the third leaf in soil drying plants with or without bacteria showed relatively high value in light period and low value in the dark period once the rate reached maximal rate (Figure 4.13). In both well watered and drying soil, 5C-2 did not show a stimulation effect on the growth of the second and the third leaves. Colonies of introduced strain 5C-2 were detected from roots of bacteria inoculated roots but not control (Figure 4.1). No significant difference was found between well watered and soil drying plants, and between experiment 1 and 2.

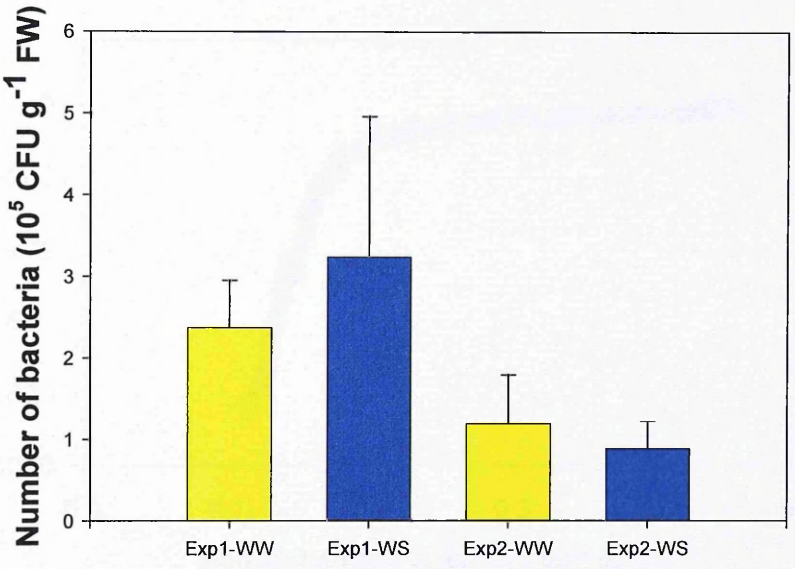

Figure 4.1. Number of bacteria (*V. paradoxus* 5C-2) isolated from roots of wheat plants in experiment 1 with well watered (Exp1-WW) or soil drying (Exp1-WS); and experiment 2 with well watered (Exp2-WW) or soil drying (Exp2-WS). No bacteria colony was detected from control plants which were not inoculated by *V. paradoxus* 5C-2. Data are means ± SE of 3 replicates.

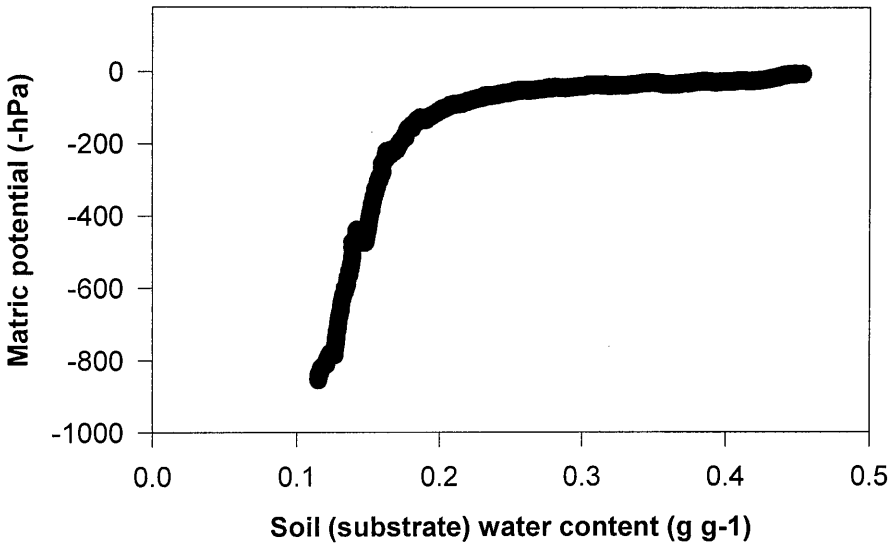

Figure 4.2. Relationship between soil (substrate) matric potential and gravimetric water content for the substrate used in this study.

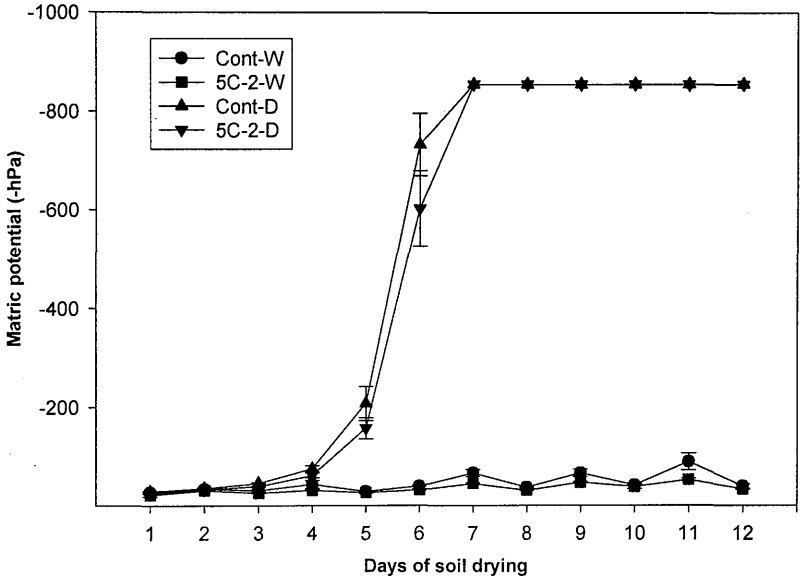

Figure 4.3. Experiment1: Matric potential of soil (substrate) in four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Each pot was weighed every afternoon before watering. Soil drying was applied by withholding water. Data are means ± SE of 5 replicates.

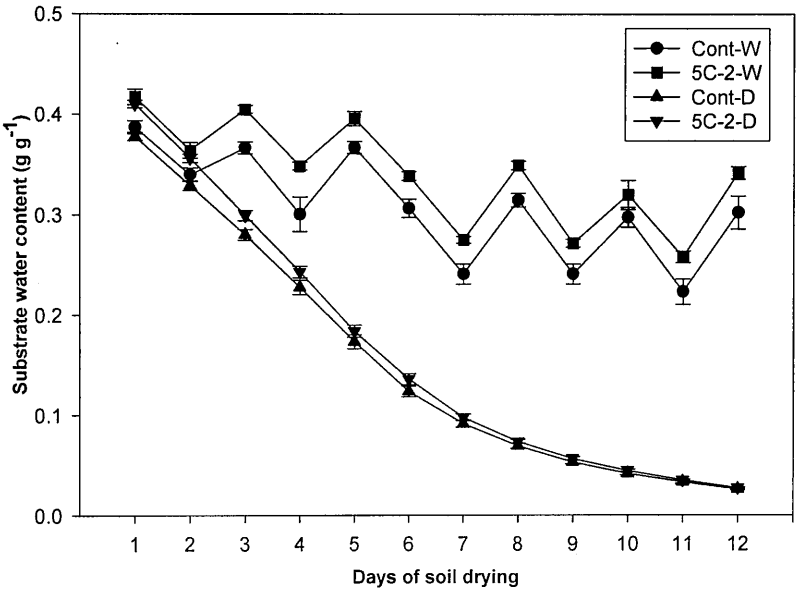

Figure 4.4. Experiment1: Substrate water content of soil subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Each pot was weighed every afternoon before watering. Soil drying was applied by withholding water. Data are means ± SE of 5 replicates.

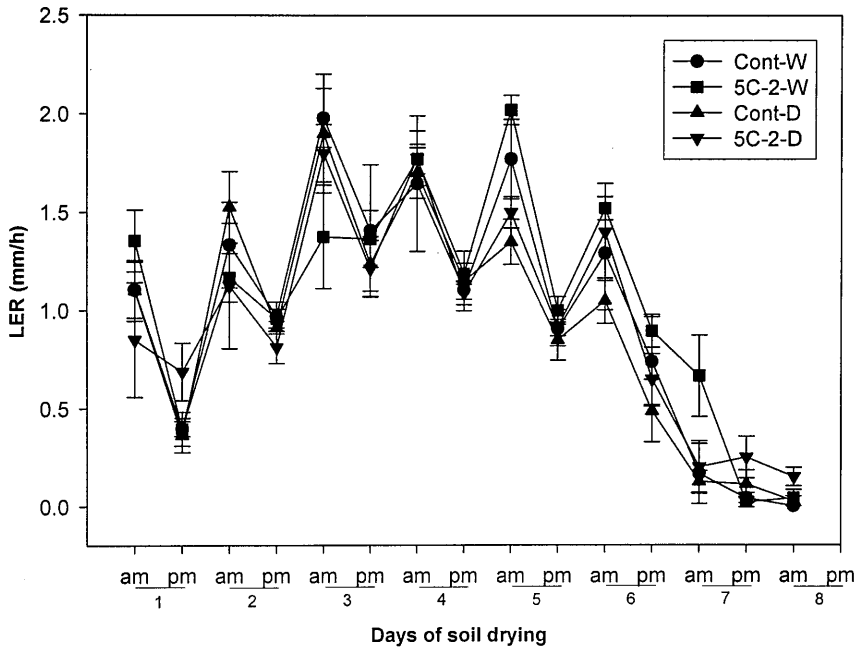

Figure 4.5. Leaf elongation rate of second leaf from wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 5 replicates.

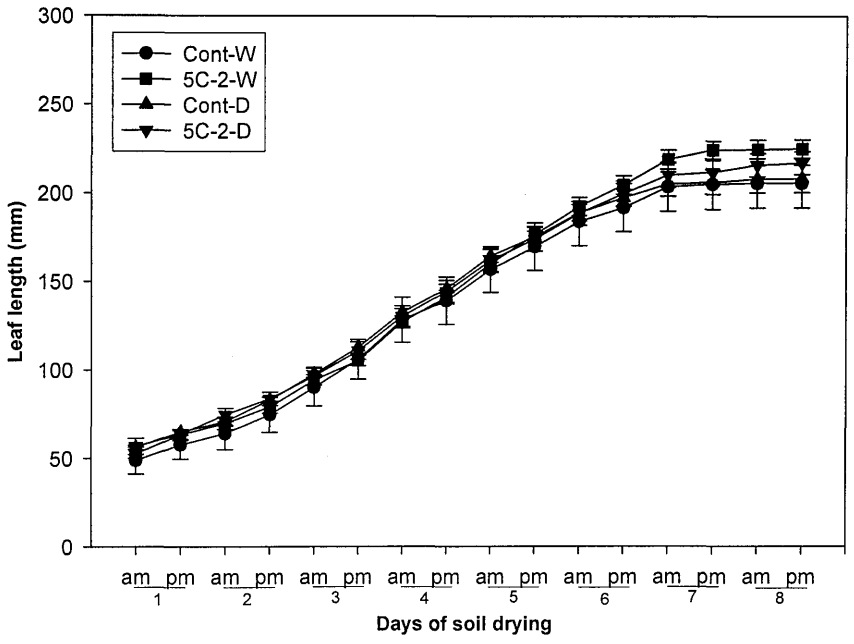

Figure 4.6. Second leaf length of wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 5 replicates.

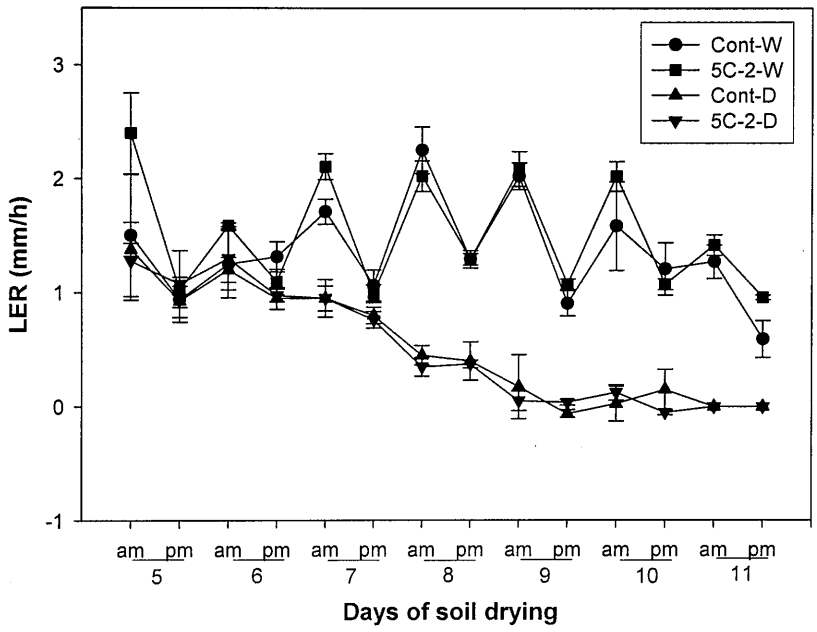

Figure 4.7. Leaf elongation rate of third leaf from wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 5 replicates.

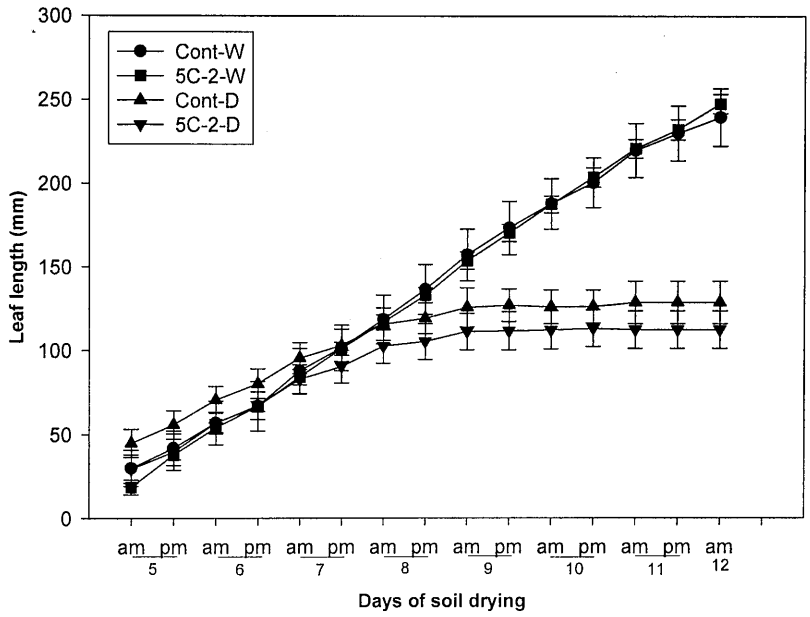

Figure 4.8. Third leaf length of wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 5 replicates.

Table 4.1. Leaf length (mm) and LER (mm h^{-1}) ± S.E. of the third leaf at different light period points in the drying cycle when growth analyses took place. Five replicas were used for each treatment. Significance of difference was tested between well watered and soil drying treatments in control or 5C-2 inoculated plants. ** were indicated as significance at the 5% level, ***were indicated as significant at the 1% level.

	Well watered for 9 days	9 days drying	Well watered for 11 days	11 days drying
Leaf length	173±16	127±10 **	230±16	129±13 ***
LER	2.02±0.12	0.175±0.28 ***	1.27±0.14	0 ***
	Well watered with 5C-2 inoculated	5C-2 + 9 days drying	Well watered with 5C-2 inoculated	5C-2 + 11 days drying
Leaf length	170±5	112±11 ***	232±6	113±11 ***
LER	2.08±0.15	0.05±0.08 ***	1.42±0.09	0 ***

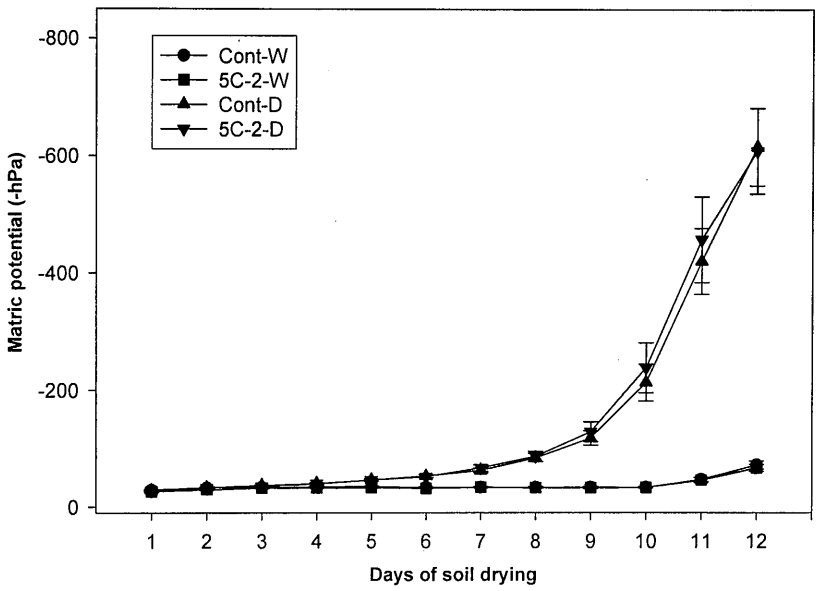

Figure 4.9. Experiment 2: Matric potential of soil (substrate) subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Each pot was weighed every afternoon before watering. Soil drying was imposed by supplying plants daily with 50% of the water that was lost through evapo-transpiration (Eta) in the previous 24 hours. Data are means ± SE of 11 replicates.

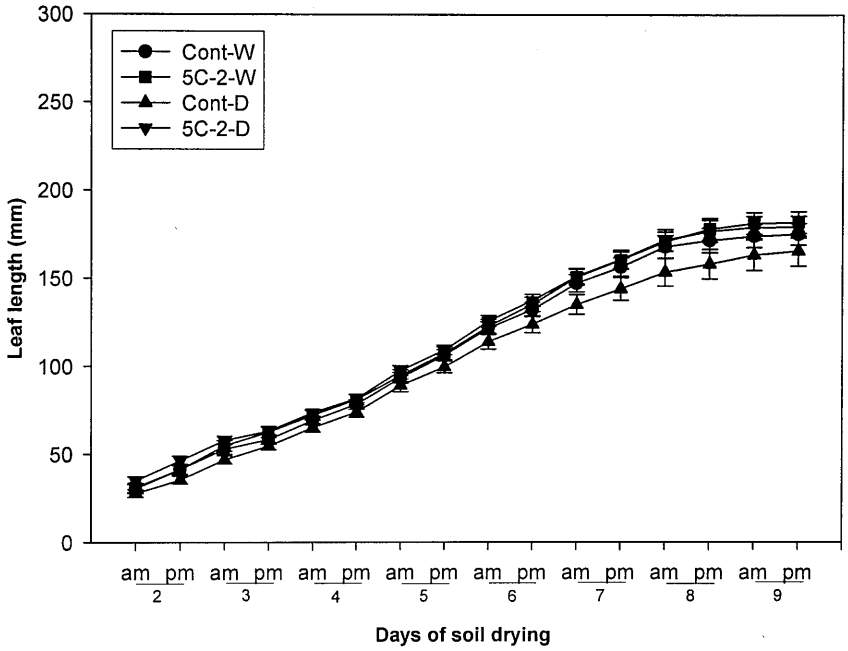

Figure 4.10. Second leaf length of wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 11 replicates.

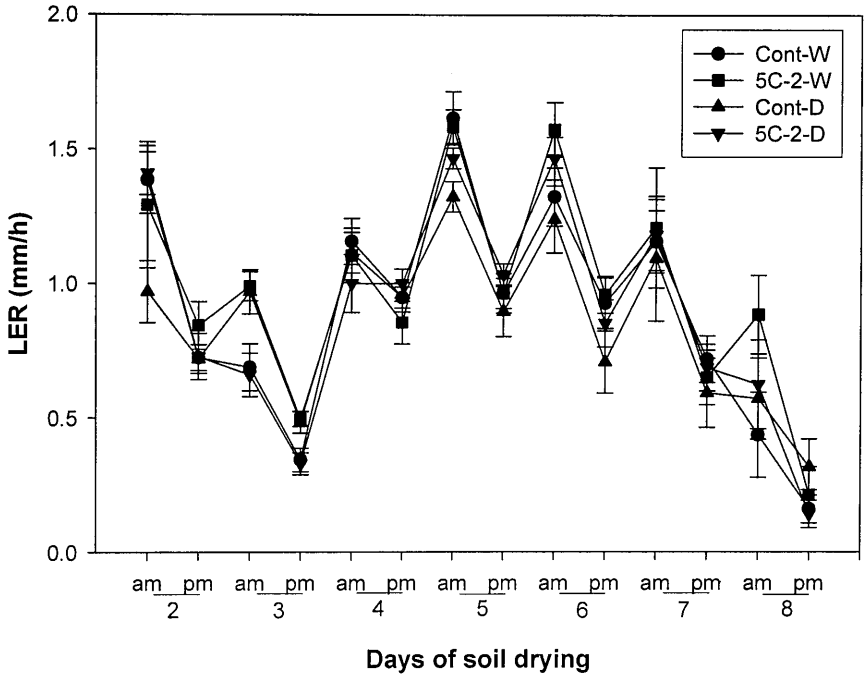

Figure 4.11. Leaf elongation rate of second leaf from wheat plants subjected to four treatments: control well watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 11 replicates.

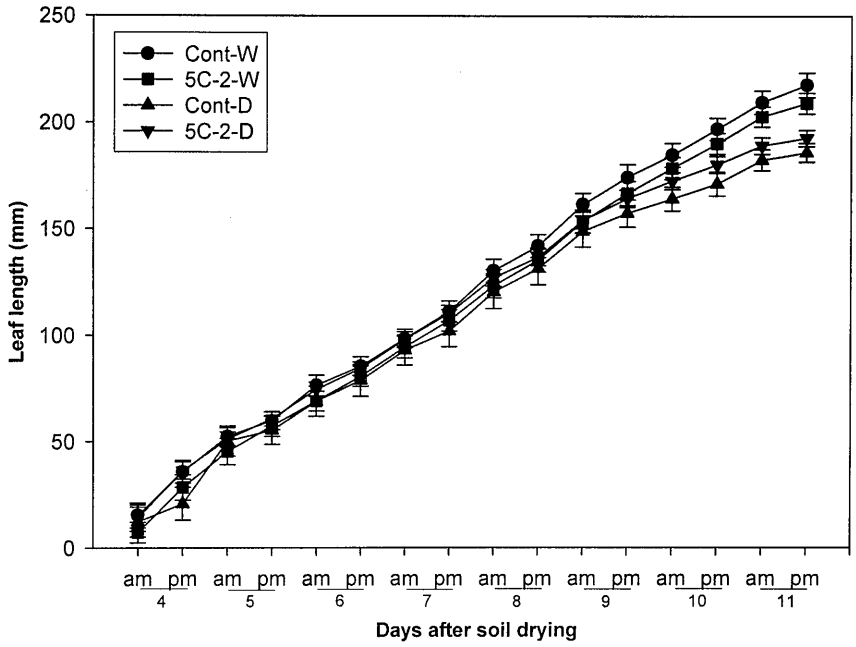

Figure 4.12. Third leaf length of wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 11 replicates.

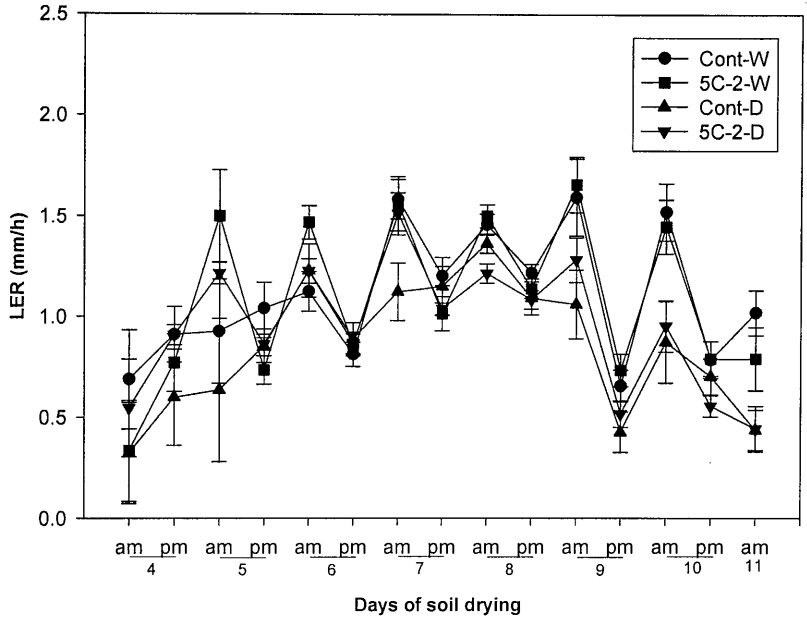

Figure 4.13. Leaf elongation rate of third leaf from wheat plants subjected to four treatments: control watered well (Cont-W ●); control with soil drying (Cont-D ▲); 5C-2 inoculated plants well watered (5C-2-W ■); 5C-2 inoculated with soil drying (5C-2-D ▼). Leaf length was measured when photoperiod started (am) and before it finished (pm). Data are means ± SE of 11 replicates.

Table 4.2. Leaf length (mm) and LER (mm h^{-1}) ± S.E. of the third leaf at different growth time in the drying cycle when growth analyses took place. Eleven replicas were used for each treatment. Significance of difference was tested between well watered and soil drying treatments in control or 5C-2 inoculated plants. ** were indicated as significant at the 5% level, ***were indicated at significant at the 1% level.

	Control 11 days well watered	11 days drying	5C-2 inoculated + 11 days well watered	5C-2+11 days drying
Leaf Length (mm)	217±6	186±4 ***	209±5	193±4 **
	control	9 days drying	5C-2 inoculated	5C-2+9 days drying
LER (mm h^{-1})	1.59±0.19	1.06±0.17 **	1.65±0.14	1.28±0.11 **

4.4 Discussion

In control or bacterial inoculated plants grown well supplied with water, increased growth (LER) during the light period was observed in the second and third leaves either in experiment 1 or 2 (Figure 4.5, 4.7, 4.11, 4.13). Although enhanced growth rate during the dark period was reported in tall fescue (Durand et al., 1995, Parrish and Wolf, 1983), higher growth rate during light period was also noted in wheat by Dodd & Davies (1996). The LER has been suggested to be sensitive to VPD (Squire et al., 1983). Decreased water potential in the elongating cells of grass due to the transpirational water loss exceeding water uptake during the light period, plants could develop water stress despite high levels of soil water (Watts, 1972). The enhanced growth during the light period suggests hydraulic signalling is unlikely to be the only factor in controlling plant growth at this time. To validate this, water status of growing cells in leaves during light and dark period needs to be studied in any future experiment. Another factor, sugar which was suggested to be important in the growth of wheat leaves (Kemp, 1981) could be a factor which can enhance growth during light period.

In experiment 1, complete cessation of watering was applied to plants and this resulted in a sharply decreasing leaf growth rate of the third leaf (Figure 4.8 and 4.7), although the second leaf did not show any reduction in LER or limitation in leaf length, which may be because the leaf approached maturily when the drying cycle started. There was no pronounced diurnal rhythm found in the third leaf of un-watered plants (Figure 4.7). Belimov et al. (2009 b) observed a growth promotion effect of 5C-2 on pea plants in soil drying conditions (40% of ET applied daily). It seems possible that daily soil drying with re-watering cycles may be important to allow bacterial effects on leaf growth, as under such conditions, chemical signalling is likely to be of most importance. Therefore, a mild soil drying (deficit irrigation) was imposed on wheat

seedlings in experiment 2.

In experiment 2, 50% of water lost through evapo-transpiration in the previous 24 hours was given back to plants daily. The third leaf showed gradually decreasing length and LER but still grew (Figure 4.12, 4.13). In the light period of 9 days after soil drying, a significant difference was found between well watered and soil drying plants either bacterial-inoculated or controls, but a similarly enhanced diurnal rhythm was also found in the third leaf of plants subjected to soil drying (Figure 4.13), indicating hydraulic signalling is not the limiting factor of growth in soil drying plants. However in either well watered and soil drying plants of experiment 1 and 2, no significant stimulation of growth by 5C-2 was observed. Although in the well water treatment, the third leaf reached maximal growth rate 2 days earlier in bacterial inoculated plants than in control plants, no significant difference was observed in leaf length between control and inoculated plants (Figure 4.11, 4.12). Bacterial colonisation of the root system detected at the end of experiments was approximately 10 times less than peas grown in a similar substrate or tomatoes grown in gnotobiotic conditions (Belimov et al., 2007, Belimov et al., 2009 b) or *Arabidopsis* (discussed in chapter 3). Although it was shown that colony number is not important in promoting root growth by the gnotobiotic assay (Belimov et al., 2007), the plant used here was grown in pots with an unsterile substrate. Bacteria or fungus in this substrate could bring competition with inoculated 5C-2. Under this condition, bacteria population could become important for survival and functioning of 5C-2.

Different rhizobacterial strains may have favoured hosts. *V. paradoxus* 5C-2 was isolated from Indian mustard (*Brassica juncea L. Czern*) (Belimov et al., 2005) rather than wheat. This is the first study of the interaction between 5C-2 and wheat. Several reports show that growth promotion by bacteria containing ACC deaminase varies

from strain to strain in wheat (Naveed et al., 2008, Nadeem et al., 2010, Zahir et al., 2009). Zahir et al. (2009) showed that 10 ACC deaminase containing rhizobacteria strains were isolated from wheat rhizosphere had varying growth promotive effects on wheat in axenic conditions with salinity stress. Three strains (*Pseudomonas putida*, *Pseudomonas aeruginosa*, *Serratia proteamaculans*) out of 10 strains showed most promising effects compared to control, but no significant difference was observed among these three strains in the root or shoot growth. In pot experiments with salinity stress, the same three strains showed promotion effects on growth and yield of wheat, particularly, *Pseudomonas putida* was most effective one compared to other two strains. Nadeem et al. (2010) screened 18 rhizobacteria containing ACC deaminase strains and found 4 strains effectively stimulated growth of wheat under high salinity conditions *in vitro*. The greatest growth stimulation was observed in with two strains. Therefore, *in vitro* screening could be useful to select the most effective strains in promoting wheat growth before moving to pot experiments.

In this study, plants less than two weeks old were used rather than mature plants used by Belimov et al. (2009 b). At different development stages, plants can regulate or respond to hormone signals differently. It was found that the expression of ethylene response genes was enhanced in proliferating and expanding leaves but not mature leaves under osmotic stress conditions in *Arabidopsis* (Skirycz et al., 2010). Expression of ABA synthesis and response genes were differently regulated (up or down) in mature leaves under osmotic stress comparing to control. These reports and others indicate that ethylene can be crucial in regulating the response of young tissues to environmental stresses. In the present study, it seems that young plants may use different mechanisms to regulate the response to ethylene, compared to mature plants. Attempts were made to measure foliar ethylene emission but ethylene concentration can not be detected by the method and cultivar used here.

Furthermore, ethylene could act as an earlier signal to help young tissue protect itself from stresses. The decrease of ethylene production by bacteria may negatively affect the response of young tissue to stresses. Or under stress conditions, ethylene production is strongly increased or signal transduction is highly active in young tissues. Thus even though bacteria may decrease root-sourced ethylene levels, this decrease may not be important in the regulation of the responses of young tissue to stresses. These suggestions raise an interesting question, how different tissues response to stresses and bacterial inoculation, and whether ethylene acts differently in different leaf developmental stages. To further explore these questions, in the next chapter, mature wheat plants were used and responses of different leaves to soil drying were investigated. Since many factors contribute to growth regulation, a specific response (stomatal response) was studied in the next chapter.

Chapter 5 – The role of ethylene in regulating leaf age-dependent stomatal responses to ABA and soil drying

5.1 Introduction

Abscisic acid (ABA) is a key signal involved in regulating plant responses to stress, including drought, high VPD, heat stress, salinity stress, and nutrient deficiency (Wilkinson and Davies, 2002). ABA synthesised by leaves and/or transported from roots can close stomata, consequently plants can save water by reducing transpiration to adapt to stress conditions such as drought. Although ABA is generally accepted as an anti-transpirant factor (Davies and Kozlowski, 1975, Davies and Zhang, 1991, Jones and Mansfiel.Ta, 1970, Trejo and Davies, 1991), under some circumstances, stomata show relatively insensitive responses to ABA or to stresses that act through it (Wilkinson and Davies, 2009, Atkinson et al., 1989).

Atkinson et.al (1989) suggested that stomatal responses of wheat leaves to ABA are variable, dependent on leaf age. Stomata of more mature "aged" leaves of wheat, responded less sensitively to ABA than younger leaves. However, fundamental understanding of a reported insensitivity of the stomatal response to ABA in aging leaves is lacking and implications of this phenomenon have not been extensively explored. Furthermore, it was pointed out in the work of Atkinson et.al (1989) that photosynthesis was dramatically reduced (over 60%) as leaves matured (plant grew from 25 days to 35 days), but that similar water loss was found throughout the growth period (plant grew from 25 days to 35 days). This leads to decreased water use efficiency when leaves age (Atkinson et al., 1989). One general observation made historically by farmers and gardeners alike has been that in many herbaceous plants

exposed to stress, old leaves wilt first, followed by younger leaves, whilst rapidly expanding leaves wilt last (Raschke and Zeevaart, 1976). Zhang & Davies (1989) also reported young sunflower leaves exhibited higher water relations than old leaves when plants were exposed to soil drying. This could be explained by a differential stomatal response to a reduction in water availability, and/or other stomatal closing stimuli. Experiments described here were designed to elucidate the mechanism behind the relative insensitivity of the stomatal response to ABA in aging leaves, and to elucidate mechanisms which could help to develop new approach to improve water use efficiency in agriculture practice during crop vegetative growth and improve plant quality in horticulture practice.

In addition to effects of leaf age on stomata responses to ABA, reduced sensitivity to ABA has also been found in *Leontodon hispidus* plants exposed to ozone pollution (Wilkinson and Davies, 2009). Elevated ozone concentrations (up to 70 ppb) reduced sensitivity of *Leontodon hispidus* stomata to soil drying and to exogenous foliar sprayed or stem injected ABA. However, by pre-treating with an ethylene perception antagonist 1-methylcyclopropene (1-MCP), responses of stomata to both applied ABA and soil drying were fully restored under elevated ozone concentrations, indicating that ethylene was involved in the loss of stomatal sensitivity to ABA. Many reports, including that of Wilkinson and Davies (2009), show that elevated ozone stimulates ethylene production in plants (Diara et al., 2005, Overmyer et al., 2003, Tamaoki et al., 2003, Sinn et al., 2004, Wellburn and Wellburn, 1996). In addition, Benlloch-Gonzalez et al. (2010) also noted an antagonistic effect of ethylene on drought-induced stomatal closure in K^+ -starved sunflower plants with heightened ethylene production, with nutrient starved plants exhibiting greater transpiration rates.

Previous studies on stomatal responses show that ethylene can have one of two

opposing effects on stomata. Ethylene induced stomatal closure when it was applied to *Arabidopsis* leaves in the absence of exogenous ABA when plants grew under optimal conditions (Desikan et al., 2006). However, when both ABA and ethylene were applied to epidermal peels of *Arabidopsis* plants, ethylene antagonised the effect of ABA to close stomata and stomata stayed open (Tanaka et al., 2005). The mechanism by which an ethylene signal regulates stomata or the stomatal response to ABA is still not fully understood. Hydrogen peroxide was suggested to be a linkage between ABA and ethylene signalling in guard cells (Desikan et al., 2006, Wilkinson and Davies, 2010).

As discussed in chapter 1, many stresses such as drought, disease, wounding, ozone pollution, and high temperature can stimulate ethylene biosynthesis (Morgan and Drew, 1997, Wang et al., 2002, Wilkinson and Davies, 2010). Ethylene is involved in growth modulation and in developmental processes such as leaf senescence, epinasty and abscission (Wang et al., 2004, Else and Jackson, 1998, Pandey et al., 2000). Several research groups have reported that ethylene promotes leaf senescence (Jing et al., 2005, Zacarias and Reid, 1990, Pandey et al., 2000, Munne-Bosch and Alegre, 2004). Furthermore, leaf senescence is often associated with the high levels of ethylene production (Jing et al., 2005, Zacarias and Reid, 1990). Zacarias and Reid (1990) suggested that, in addition to ethylene, ABA also promotes senescence via an ethylene independent pathway. Jing et.al (2005) reported that ethylene can play a dual function in regulating leaf senescence, both as an inducer and a repressor, depending on the time of ethylene exposure. Although it is clear that ethylene is involved in leaf senescence and that ethylene can antagonize ABA function in stomatal closure, the connection between ethylene induced stomatal insensitivity to ABA and ethylene promoted senescence has not previously been made. The work presented by Haroni and Sisler (1979) described ethylene production patterns in

different aged leaves of tobacco, and it was shown that young expanding leaves actually produced higher levels of ethylene than all other ages of leaves. When leaves were fully expanded, ethylene evolution started to decrease. When leaves began to senesce and turn yellow, ethylene levels increased again but were not as high as in expanding leaves. Even if wheat plants exhibit similar patterns of ethylene evolution as tobacco plants, ethylene may still antagonize stomatal responses to ABA through age-induced changes in ethylene sensitivity, rather than through age-induced increases in the extent of ethylene production. The hypotheses tested in this chapter are, then, that 1) the loss of stomatal sensitivity to ABA in aged leaves is due to antagonism from aging-associated changes in ethylene biosynthesis, and 2) the antagonistic effect of ethylene on stomatal responses to ABA is enhanced in the aged leaves compared to the young leaves because of increases in cellular sensitivity to ethylene rather than, or in addition to, any observed increased ethylene production.

5.2 Methods

The spring wheat (*Triticum aestivum*) cultivar Ashby, which is very popular with UK growers, was used in this study. Seeds were pre-germinated as described in chapter 2 before they were transferred to pots with growing substrate comprising a 1:1 (w/w) mixture of a loam-based compost (John Innes No. 2, J. Arthur Bowers, Lincoln, UK) and quartz sand. Pre-germinated seeds were planted carefully in pots filled with the same weight of growing substrate. Plants were raised in the green-house for 26 days and were then moved to a growth chamber. Growth conditions in the green-house and the growth chamber were as described in chapter 2. Plants were fully watered before the application of soil drying or chemical spray.

Soil drying was applied to 30 day old plants (table 5.1) by withholding water until matric potential reached around -300 –hPa calculated from the moisture release curve as described in chapter 4, after weighing pots at the end of the photoperiod. At that point, stressed plants were fully re-watered (until drops of water appeared in the holding tray of pots before the photoperiod finished). Stomatal conductance was measured the following day on abaxial and adaxial sides of leaves with a diffusion porometer (AP-4, Delta-T Devices Ltd, UK). Means of gs from abaxial and adaxial sides of leaves were calculated and presented in following graphs. Four different aged leaves (Leaf 2, 3, 6, and 7 on the main stem, numbering from the base of the plant) were used for stomatal conductance measurements.

ABA or ACC were applied to 34 days old plants which were well watered as foliar spray (table 5.1). ABA was dissolved in ethanol, and ACC was dissolved in water for stock solution preparation. Both sides of leaves were sprayed with water, ABA (10^{-7} mmol m^{-3}, 3×10^{-7} mmol m^{-3}, 7×10^{-7} mmol m^{-3}) or ACC (3×10^{-6} mmol m^{-3}, 7×10^{-7} mmol m^{-3}, 10^{-6} mmol m^{-3}). 0.025% (v/v) of a wetting agent-Silwett (L-77, De Sangosse Ltd, Cambridge, UK) was included in all solutions. ABA or ACC solutions (30 ml per plant) were sprayed on each plant 2 hours after the start of photoperiod and stomatal conductance was measured on abaxial and adaxial sides of leaves with a diffusion porometer (AP-4, Delta-T Devices Ltd, UK) 3 hours after spraying. Means of gs from abaxial and adaxial sides of leaves were calculated and presented in following graphs. A proportion of each chemical sprayed on to the leaf surfaces was assumed to penetrate the interior of the leaves.

Some plants which were well watered were pre-treated with 1-MCP (kindly provided by Smart-Fresh, AgroFresh Inc, Spring House PA, USA), applied as a sprayable liquid after dissolving a solid preparation containing 3.8% active ingredient, at a rate of 0.1 g

L^{-1} in a 0.025% (v/v) Silwett L-77 solution. Once made up, the solution (30 cm^3) was immediately sprayed over both sides of the leaf of plants which had been placed inside 0.8×0.8 m^3 cardboard boxes with 10 plants in a ventilated room. Each control plant sprayed with 30 ml 0.025% (v/v) Silwett L-77 solution only also was kept in cardboard boxes. Plants were incubated in 1-MCP (released as a gas within 5 minutes of the active ingredient being dissolved) in closed boxes for 16 h. After this time, plants were transferred to the growth chamber. Soil drying or chemical sprays were applied to plants 4 days after 1-MCP treatment (table 5.1) as described above. 1-MCP treatment can be effective at preventing ethylene binding to its receptor for up to 1 month after application (Sisler and Serek, 2003).

Bacteria, strain *Variovorax paradoxus* 5C-2, grown on BPF medium, were used to prepare liquid suspensions. Liquid suspension was applied to the plant growing substrate by thoroughly mixing with the substrate. The final bacterial concentration in the inoculated substrate was 10^6 cells g^{-1}. Pre-germinated seeds were planted carefully in the pot filled with the bacterial-inoculated growing substrate (one seed per pot). Seven days prior to initiation of the soil drying treatment described above, new bacterial solution with same concentration as earlier inoculation was used to irrigate plants.

Leaf tissues were collected from control and 5C-2 inoculated plants 16 h after rehydration. ABA concentrations were determined by using a radioimmunoassay as described in chapter 2. Three hours after ACC application, leaf tissues of different ages were collected for ACC concentration determination by GC-MS, as described in chapter 2. Leaf tissues (0.5 g FW) were also sampled 3 hours after ACC application to determine ethylene emission rate after incubating in 7.8 ml sealed glass vials to collect ethylene for GC analysis as described in chapter 2.

Table 5.1. Timetable of different treatments with rhizobacteria or chemical spray under different irrigation condition

Treatment	Growth period	Treatments start	Treatments end
ABA foliar spray	Grow 34 day under well watered conditions	34 day after sowing (DAS)	Measure gs on 34 DAS
1-MCP + soil drying	Grow 30 day under well watered conditions, pretreat 1-MCP on 26 DAS	Soil drying 30 DAS (usually 2-3 days) then rehydration	Measure gs after rehydration
5C-2 + soil drying	Grow 30 day under well watered conditions	Soil drying 30 DAS (usually 2-3 days) then rehydration	Measure gs after rehydration and collect leaf samples for further analysis
ACC foliar spray	Grow 34 day under well watered conditions	34 DAS	Measure gs on 34 DAS and collect samples for ACC or ethylene
ACC + 1-MCP	Grow 34 day under well watered conditions, pretreat 1-MCP on 30 DAS	Spray on 34 DAS	Measure gs on 34 DAS

Statistics

Pairwise comparisons used Student's t-tests and standard errors (SE) in SigmaPlot for Windows Version 7.0 (Jandel Scientific, Erkrath, Germany). One way analysis of variance (ANOVA) was performed to determine differences in stomatal conductance among the 4 age categories of leaves under different treatments (SPSS version 19 (SPSS Inc, Chicago, USA)).

5.3 Results

5.3.1 Effect of leaf age on stomatal response to applied ABA.

To investigate effects of leaf age on the stomatal response to ABA, three concentrations of ABA (10^{-7} mol m^{-3}, 3×10^{-7} mol m^{-3}, 7×10^{-7} mol m^{-3}) were applied to leaves of different ages on intact wheat plants (Figures 5.1 and 5.2). Figure 5.1 showed the absolute value of gs from 1 experiment. In figure 5.2, % of control was

used to present gs as data from more than one separated experiments were presented in one graph. ABA-induced stomatal closure was shown to be sensitive in the young mature; especially leaf 7, where even the lowest ABA concentration (10^{-7} mol m^{-3}) significantly decreased stomatal conductance (Figures 5.1 and 5.2). However, with increasing leaf age, closure became less sensitive to ABA. In Figure 5.2, leaf 3 and leaf 2 only showed (statistically insignificant) reductions of 26% and 22% respectively. The highest ABA concentration (7×10^{-7} mol m^{-3}) significantly closed stomata in leaves of all ages except the oldest tested one - leaf 2, which failed to respond significantly to any of the three concentrations of ABA used. In general, older, more mature leaves lost their ability to close their stomata in response to exogenous ABA treatment.

5.3.2 1-MCP restores the sensitivity of stomata in aged leaves to soil drying treatment.

A soil drying treatment was applied to wheat plants to produce endogenous ABA, and a rehydration treatment was applied to stimulate potential coincidental ethylene production (GomezCadenas et al., 1996). Plants were rehydrated when substrate matric potential reached -300 -hPa (Figure 5.3). Drought-induced stomatal closure was less sensitive in aged leaves (Figure 5.4). In water stressed plants, the ethylene perception antagonist 1-MCP had no effect on gs in well watered or droughted leaves which were less mature (Leaf 7 and 6), but in the more mature leaves (Leaf 3 and 2) it increased stomatal conductance in well watered plants, whilst enhancing stomatal closure in droughted plants. Thus ethylene was responsible for the lack of stomatal response to drought in more mature leaves. Inhibition of ethylene function improved stomatal responsiveness to drought in aged leaves.

5.3.3 Substrate inoculation with of *V. paradoxus* 5C-2 restores the sensitivity to soil drying of stomata in aged leaves.

The soil drying treatment (followed by a rehydration when matric potential reached -300 -hPa) (Figure 5.5) closed stomata in the two less mature leaves (Leaf 7 and 6) by 55% and 57% respectively, but did so to a lesser extent in more mature leaves, such that closure was not significant in the oldest leaf (Leaf 2) (Figure 5.6), mirroring the effect of leaf age on the stomatal response to ABA treatment (Figures 5.1 and 5.2) and soil drying (Figures 5.4). Inoculation of substrate with 5C-2 had no effect on gs of well watered or droughted plants in the less mature leaves. In the more mature leaves, 5C-2 increased gs of well watered plants but not significantly. However, bacterial inoculation reduced the gs of more mature leaves (Leaf 3 and 2) of droughted plants such that drought-induced stomatal closure was as great relatively as that exhibited by the less mature leaves. This result is consistent with the effect of 1-MCP seen in Figure 5.4, such that both modulators of ethylene restored the sensitivity of drought-induced stomatal closure in the aged leaves to that exhibited by the less mature leaves, even though one modifies ethylene production (5C-2 Figure 5.6), and one modifies ethylene perception (1-MCP Figure 5.4). These data strongly indicate that ethylene is one factor regulating the stomatal response to soil drying and/or ABA in aged leaves.

5.3.4 Effects of leaf age and bacterial inoculation on hormone generation

a) ABA.

To determine whether either leaf age or bacterial inoculation affects foliar ABA concentration, ABA concentration was measured in less mature (mixed samples from Leaf 6 &7) and more mature (Leaf 2 and 3) leaves. The data in Figure 5.7 show that

soil drying (followed by rehydration) increased ABA concentrations to the same extent (from a similar basal level in both less mature and more mature leaves), and that bacterial inoculation did not affect ABA concentrations (Figure 5.7), indicating that stomatal responses in leaves of different ages or in leaves treated with bacterial inoculation were not due to effects of age or bacteria on bulk ABA concentration in leaves.

b) Ethylene generation in relation to stomatal response

Ethylene emissions from leaves of different ages can often be quantified by GC using the method described above and in chapter 2. However, this method did not provide enough resolution to measure the small amounts of ethylene generated from wheat leaves of this variety. Instead, concentrations of the ethylene precursor, ACC, were determined in leaf 3 and 6 (Figure 5.10). Younger leaves contained slightly (but not significantly) higher ACC concentrations than old leaves, indicating that the low sensitivity of stomatal responses to ABA in older leaves is not due to greater production of ethylene or of its precursor ACC. Thus it is necessary to determine whether leaf age affects stomatal sensitivity to ethylene, in order to explain the ability of 1-MCP and 5C-2 to restore stomatal sensitivity to ABA in older leaves (Figures 5.4 and 5.6).

5.3.5 Effects of leaf age on stomatal sensitivity to applied ACC.

To investigate the possibility that leaf age affects stomatal sensitivity to ethylene, the ethylene precursor -ACC was applied to intact wheat plants as a foliar spray. Responses of stomata in leaves of four different ages were tested after three different ACC concentrations (3×10^{-7} mol m^{-3}, 10^{-6} mol m^{-3}, and 3×10^{-6} mol m^{-3}) were applied (Figure 5.8). None of these ACC concentrations affected gs in less mature

leaves (Leaf 7 and 6). However, ACC applications decreased gs in more mature leaves (Leaf 3 and 2), especially when applied at 3×10^{-6} mol m^{-3} ACC. To confirm that the stomatal closing response to ACC application is due to ethylene rather than to ACC itself, plants were pretreated with 1-MCP. Stomata lost their sensitivity to the ACC application after 1-MCP pre-treatment (Figure 5.9). Following foliar application of 3×10^{-6} mol m^{-3} ACC, concentrations of both ACC and ethylene emission were increased equally in younger and older leaves (Figures 5.10 and 5.11). These results indicate that stomata of older leaves are more sensitive to ethylene generated by an ACC application than younger (newly mature) leaves.

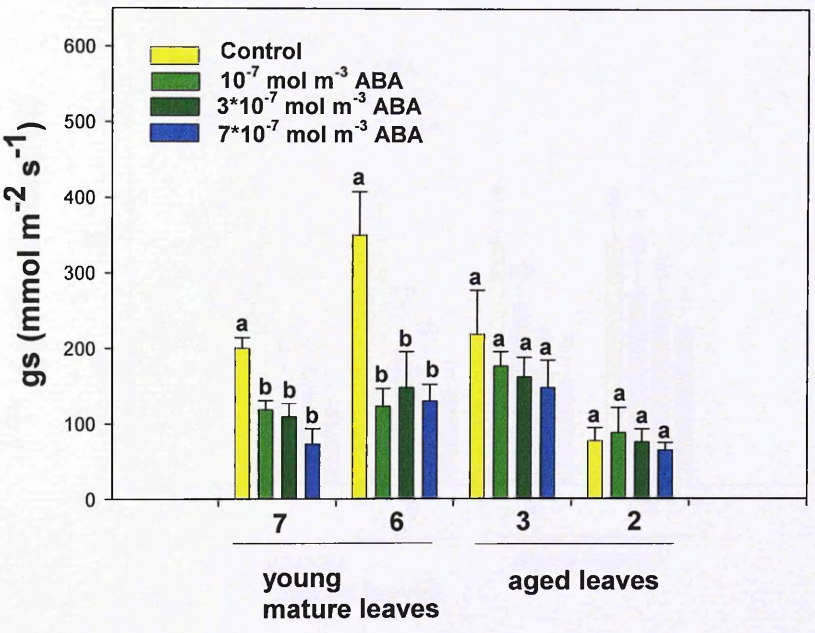

Figure 5.1. Stomatal conductance (gs) of four leaves of different ages following foliar ABA application. Plants were well watered. Three concentrations of ABA (10^{-7} mol m^{-3}, 3×10^{-7} mol m^{-3}, 7×10^{-7} mol m^{-3}) were applied respectively. Stomatal conductance was measured 3 hours after ABA application. Data are expressed from 5-6 replica plants within experiment. Bars indicate ± standard error (SE). Differences between treatments within leaves of a single age are denoted by different letters ($P<0.05$ Tukey test).

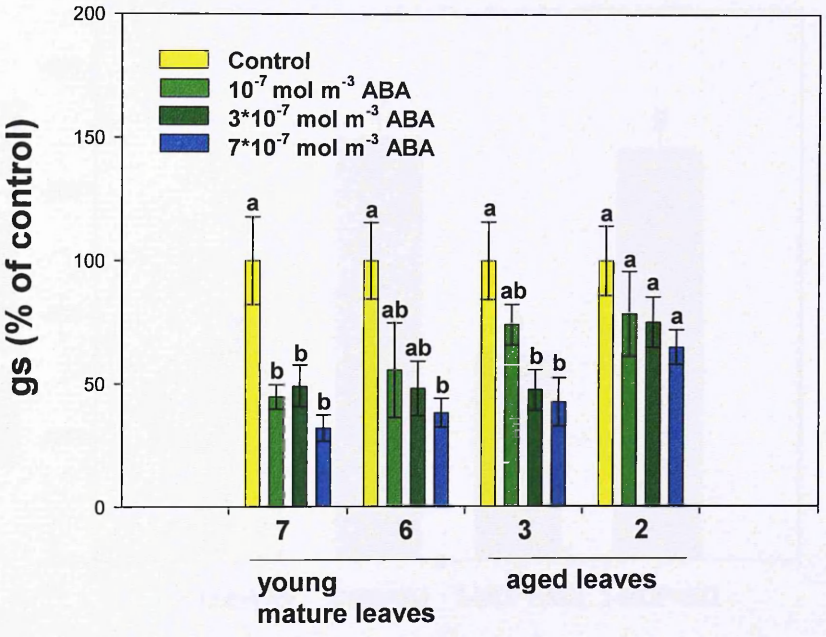

Figure 5.2. Stomatal conductance (expressed as a percentage of the control treatment) of four leaves of different ages following foliar ABA application. Plants were well watered. Three concentrations of ABA (10^{-7} mol m^{-3}, 3×10^{-7} mol m^{-3}, 7×10^{-7} mol m^{-3}) were applied respectively. Stomatal conductance was measured 3 hours after ABA application. Data are from 15-16 replica plants across 3 separate representative experiments. Bars indicate ± standard error (SE). Differences between treatments within leaves of a single age are denoted by different letters ($P<0.05$ Tukey test).

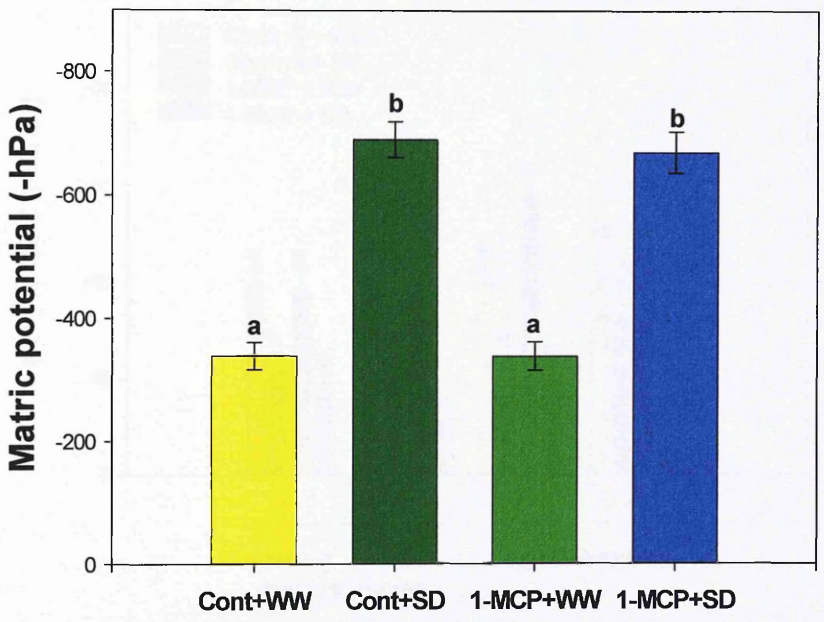

Figure 5.3. Matric potential of substrate that was watered well – WW or soil drying – SD. Plants were treated with 1-MCP or without - Cont. Before the rehydration pots were weighed to calculate matric potential as described in chapter 4. Experiments were repeated 3 times and data here are from a representative experiment. Bars indicate ± standard error (SE) (n=7 or 8). Differences between treatments are denoted by different letters ($P<0.05$ Tukey test).

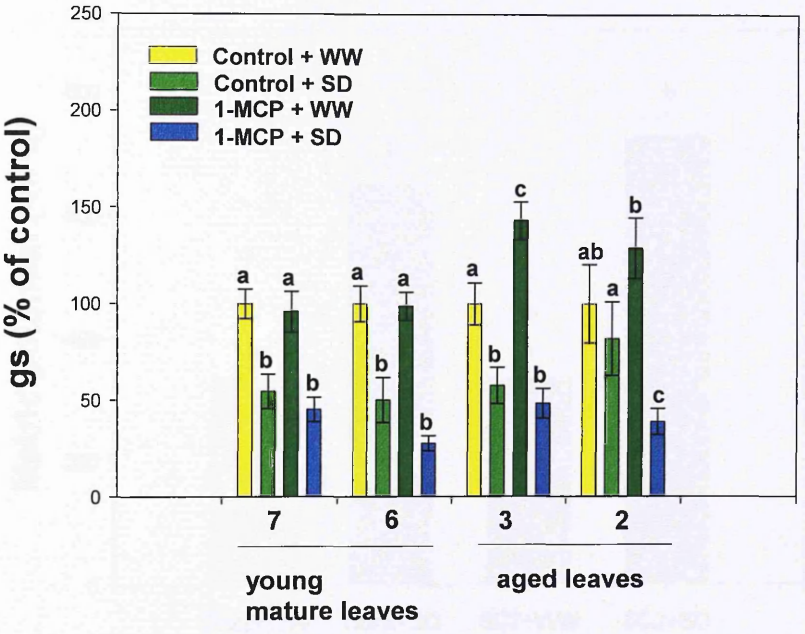

Figure 5.4. Stomatal conductance (expressed as a percentage of the control treatment) of four leaves of different ages on well watered – WW or soil drying – SD plants. Measurements were taken 16 hours after rehydration with or without 1-MCP treatments. Plants (26 days old) were pre-treated by 1-MCP, then soil drying was applied 4 days after 1-MCP exposure. Data are from 15-16 replica plants across 2 separate representative experiments. Bars indicate ± standard error (SE). Differences between treatments within leaves of a single age are denoted by different letters ($P<0.05$ Tukey test).

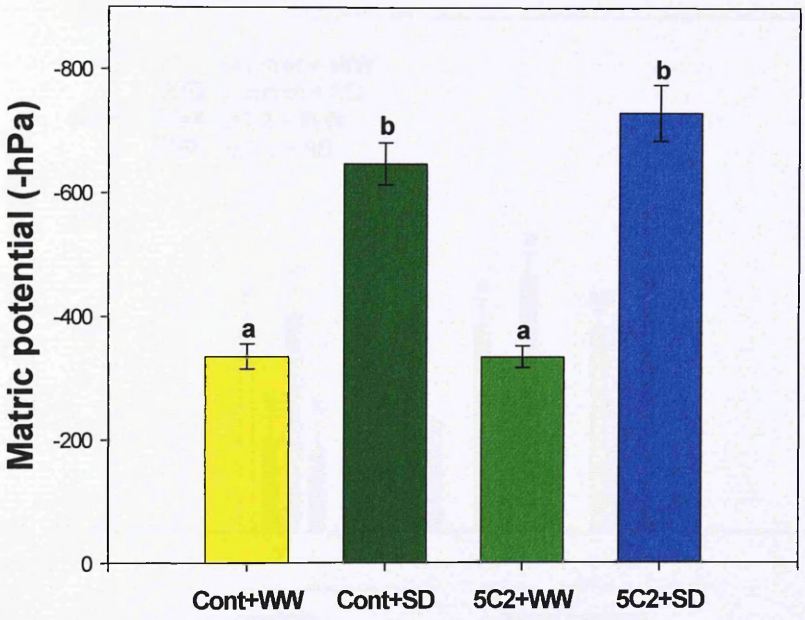

Figure 5.5. Matric potential of substrate that was watered well – WW or soil drying – SD. Plants were inoculated with *V. paradoxus* 5C-2 or without - Cont. Before the rehydration pots were weighed to calculate matric potential as described in chapter 4. Experiments were repeated 3 times and data here are from representative experiment. Bars indicate ± standard error (SE) (n=7). Differences between treatments are denoted by different letters ($P<0.05$ Tukey test).

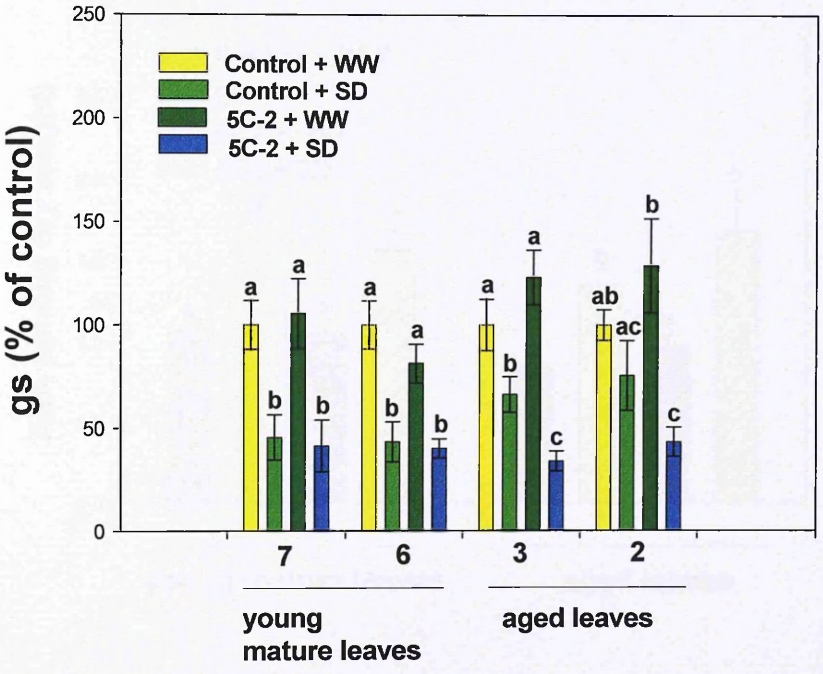

Figure 5.6. Stomatal conductance (expressed as a percentage of the control treatment) of four leaves of different ages on well watered – WW or soil drying– SD plants. Measurements were taken 16 hours after rehydration with or without *V. paradoxus* 5C-2. *V. paradoxus* 5C-2 was inoculated to plant roots at sowing time and 23 days after sowing. Soil drying was applied to 30 days old plants. Data are from 15-17 replica plants across 2 separate representative experiments. Bars indicate ± standard error (SE). Differences between treatments are denoted by different letters ($P<0.05$ Tukey test).

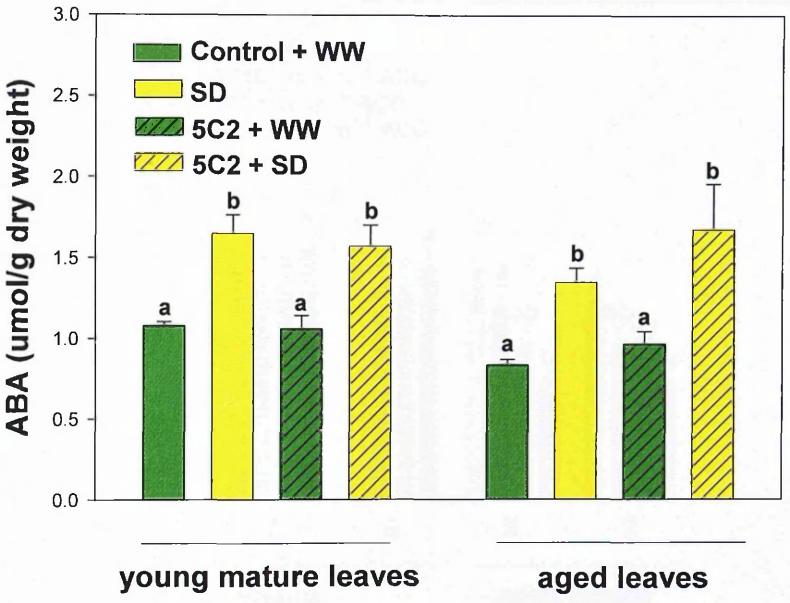

Figure 5.7. ABA contents of less (Leaf 6 and 7) and more mature (Leaf 2 and 3) leaves on well watered – WW or soil drying – SD plants; Plants were inoculated with *V. paradoxus* 5C-2 or without – control. Leaf samples were collected after measurements of stomatal conductance. Experiments were repeated 3 times and data here are from representative experiment. Bars indicate ± standard error (SE) (n=8). Differences between treatments within single leaf age are denoted by different letters ($P<0.05$ Tukey test).

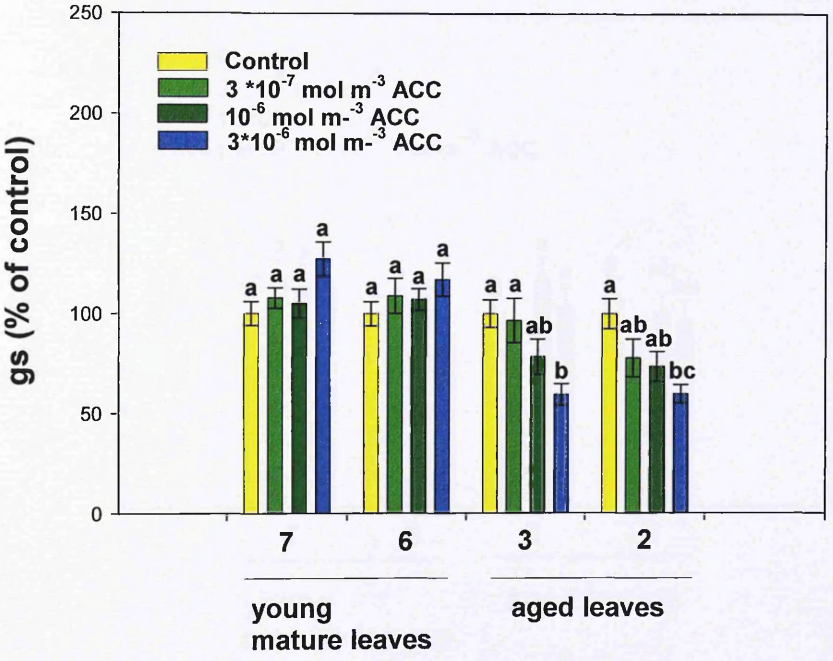

Figure 5.8. Stomatal conductance (expressed as a percentage of the control treatment) of four leaves of different ages following ACC applications. Three concentrations of ACC (3×10^{-7} mol m^{-3}, 10^{-6} mol m^{-3}, and 3×10^{-6} mol m^{-3}) were applied respectively. Stomatal conductance was measured 3 hours later after ACC applications. Data are from 20-24 replica plants across 3 separate experiments. Bars indicate ± standard error (SE). Differences between treatments within single leaf age are denoted by different letters ($P<0.05$ Tukey test).

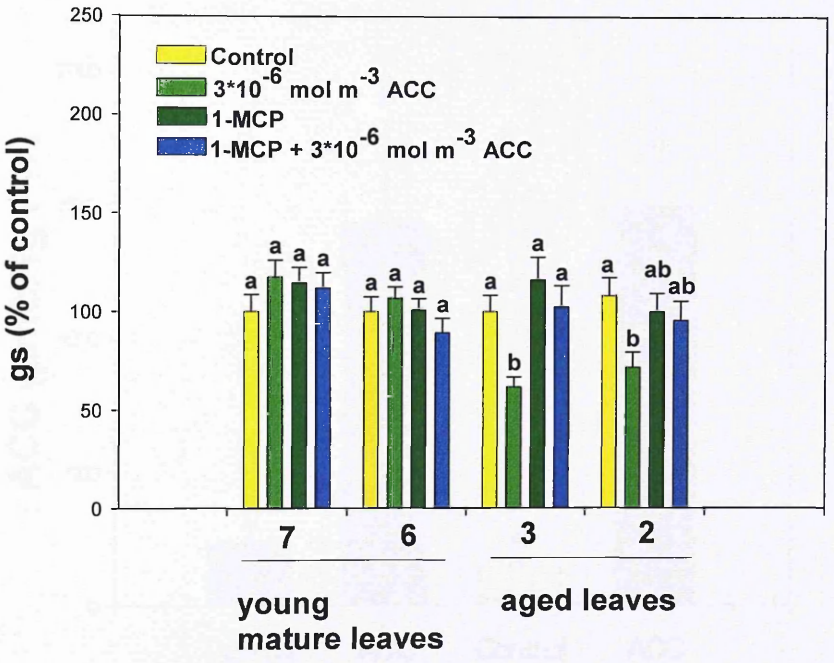

Figure 5.9. Stomatal conductance (expressed as a percentage of the control treatment) of four leaves of different ages following by 3×10^{-6} mol m^{-3} ACC applications with 1-MCP pre-treatment or without - control. Plants (30 days old) were pre-treated with 1-MCP and ACC was applied 4 days after 1-MCP treatment. Data are expressed as % of controls from 15-17 replica plants across 2 separate representative experiments. Bars indicate ± standard error (SE). Differences between treatments within single leaf age are denoted by different letters ($P<0.05$ Tukey test).

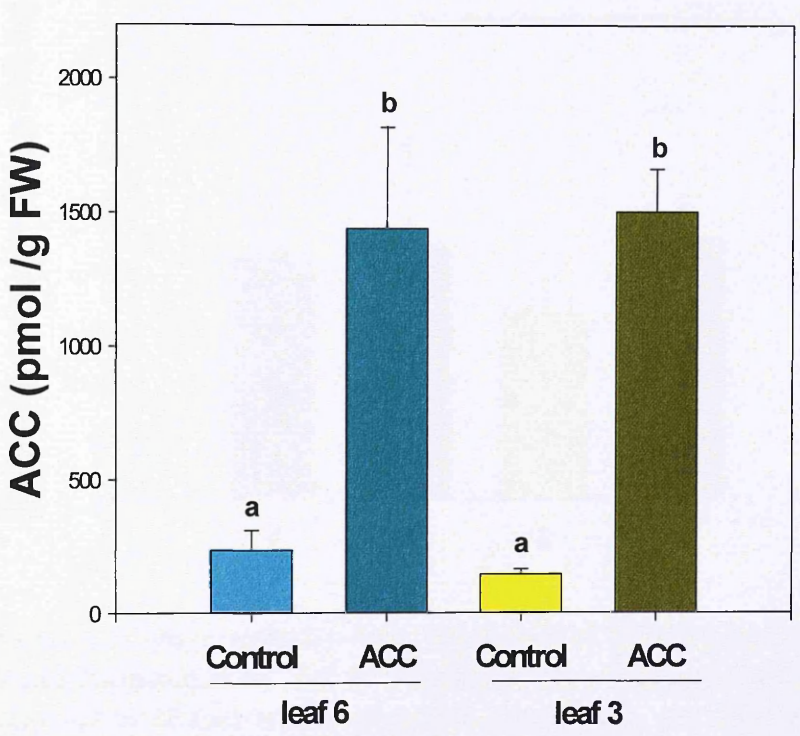

Figure 5.10. ACC concentrations of leaf 6 and 3 from 34 days old plants grown under well watered conditions following by 3×10^{-6} mol m^{-3} foliar ACC application. Leaf samples were collected 3 hours after ACC application for ACC measurement. Data are from representative experiment. Bars indicate ± standard error (SE) (n=6). Differences between treatments between two leaves are denoted by different letters ($P<0.05$ Tukey test).

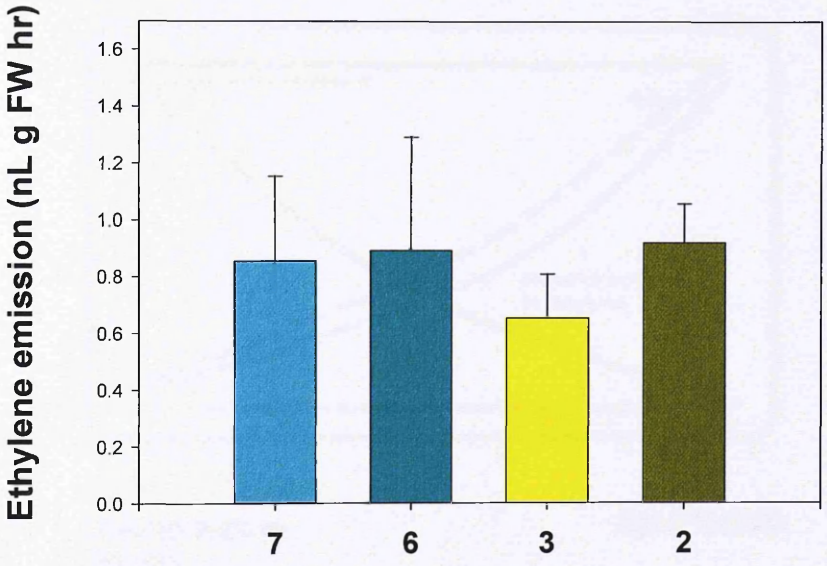

Figure 5.11. Ethylene emission from four leaves of different ages from 34 days old plants following 3×10^{-6} mol m^{-3} ACC application. Ethylene emission could not be detected in all four leaves of control plants. Data are from representative experiment. Bars indicate ± standard error (SE) (n=6).

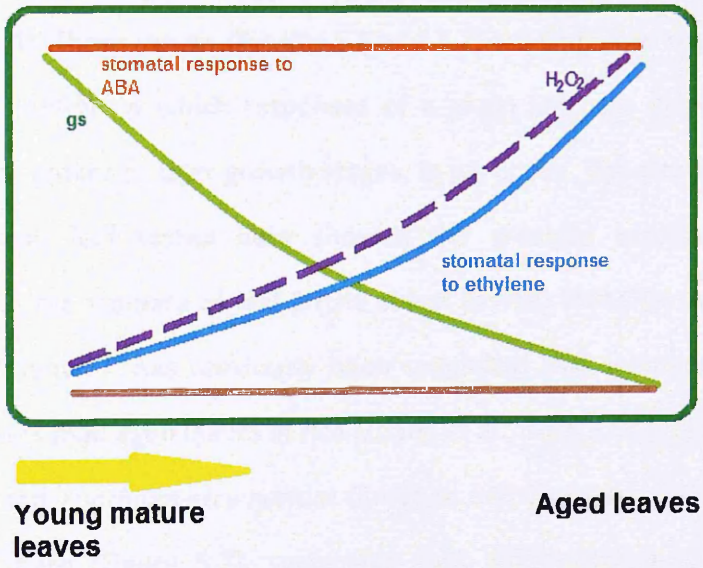

Figure 5.12. Summary of gs, ethylene, ABA, and hydrogen peroxide across different age leaves based on the data from work here.

5.4 Discussion

Here we have explored stomatal responses of mature leaves of four different ages to ABA, applied exogenously or generated endogenously by soil drying. It was found that older, more mature leaves exhibited a reduced sensitivity of the stomatal closing response to both exogenous ABA treatments and to soil drying-induced ABA (Figures 5.1, 5.2, and 5.4). These results (Figures 5.1 and 5.2) are similar to those described by Atkinson et.al (1989), in which responses of a given leaf to xylem-supplied ABA were studied at earlier or later growth stages. In particular, the stomata of leaf 7 the youngest mature leaf tested here showed the greatest sensitivity to all ABA treatments, but the stomata of leaf 2 (the oldest tested) failed to respond to any of the ABA treatments. It has previously been suggested that younger leaves contain higher ABA levels than aged leaves in rice (Zhang et al., 2009), *Coleus blumei* (LaMotte et al., 2002), and *Xanthium-strumarium* (Raschke and Zeevaart, 1976), but this was not the case here (Figure 5.7), suggesting that differences in endogenous ABA concentrations were not the reason for the age-dependent differences insensitivity to applied ABA.

There are several potential implications of age-induced differences in stomatal sensitivity to ABA. Increased sensitivity to ABA in young leaves has been suggested could contribute to turgor maintenance, which may be important for the continued expansion of young leaves in stressful conditions such as soil drying (Raschke and Zeevaart, 1976). However, water relations of the different aged leaves were not examined here. It will be important to investigate this in future experiments to explore the consequences of the loss stomatal sensitivity to ABA in aging leaves on whole plant physiology, particularly in plants experiencing various types of stress which generate or re-circulate ABA (or ethylene-see below). Furthermore, we propose here that the loss of sensitivity to ABA in aging leaves could contribute to

developmentally-induced senescence, potentially by affecting leaf water relations. This may be particularly noticeable in stressed plants (described in introduction). Vegetative senescence is an important process in plant ontology as consequences for fruit ripening and reproduction (Munne-Bosch and Alegre, 2004).

We tested the hypothesis that the loss of sensitivity of the stomatal closure response to ABA in aging leaves resulted from an effect of leaf aging on ethylene biology. Wilkinson & Davies (2010) summarize current knowledge regarding the interactions between ethylene and ABA, and proposed that ethylene plays two separate but inter-changeable roles in regulating stomatal response: when ABA levels are relatively low or are less responsive, ethylene can close stomata; however when ABA levels increase, ethylene antagonizes the effect of ABA to close stomata (Tanaka et al., 2005) such that they remain open, or re-open, depending on the chronology of the changes in hormone concentration. Therefore, ethylene could potentially be a factor which reduces ageing leaf responsiveness to ABA, as shown in Figures 5.1 and 5.2.

To investigate the role of ethylene in regulating stomatal responses of leaves of different growth stages to ABA, two different methods of manipulating ethylene biology were employed: 1) 1-methylcyclopropene (1-MCP), which can antagonize ethylene receptors and thus decrease ethylene sensitivity of plants (as described in the introduction), was applied to the plants as a foliar spray; and 2) plant growth-promoting rhizobacteria *V. paradoxus* 5C-2, which contains ACC deaminase and decrease concentrations of the ethylene precursor ACC in the xylem (Belimov et al., 2009 b) and shoots (see chapter 3), and hence the increased production of shoots (Belimov et al., 2009 b, Belimov et al., 2009 a) was applied as a soil treatment. Gomez-Cadenas et al. (1996) showed that low water potential of soil stimulated ACC production in *Cleopatra mandarin* roots, and then rehydration allowed delivery of

this ACC from roots to shoots via the transpiration stream, giving rise to a burst of ethylene production in the shoot. Inoculation of the soil around wheat roots with *V. paradoxus* 5C-2, is assumed to decrease ACC accumulation in the root when soil dries, and eventually to decrease ACC in the xylem and delivery to the shoot (Belimov et al., 2009 b), thus potentially decreasing ethylene production in shoots. In these experiments (Figures 5.4 and 5.6), soil drying (followed by some rehydration) was used to induce internal ABA production (in conjunction, presumably, with maximal ACC delivery to the shoot), and it was found that neither leaf age nor bacterial inoculation affected basal or drought-enhanced ABA concentrations (Figure 5.7). Comparing Figures 5.4 and 5.6 with Figures 5.1 and 5.2, it can be seen that soil drying had a similar effect on stomatal closure to that resulting from external ABA application, whereby less mature leaves exhibited sensitive stomatal closure, but more mature leaves exhibited a reduced sensitivity to soil drying, especially in the oldest leaf (leaf 2) tested. By pre-treating plants with 1-MCP (Figure 5.4) or after rhizobacterial inoculation (Figure 5.6), the ability of the more mature leaves to close their stomata after soil drying treatments, was restored. These experiments suggest that ethylene is an important the factor which reduces stomatal responsiveness to ABA as leaves age.

Furthermore this work indicates that rhizobacateria which contain ACC deaminase can be used as a tool for probing the involvement of ethylene in plant responses. Although 1-MCP, aminoethoxyvinylglycine (AVG), and silver ions are frequently used to study ethylene functions in plants, the use of some of these compounds can be accompanied by toxic effects. ACC-deaminase bacterial inoculation offers a relatively non-toxic and more subtle regulation of ethylene accumulation in both roots and shoots (1-MCP is only effective in disrupting shoot ethylene biology as it cannot penetrate the soil). ACC-deaminase containing rhizobacteria have been shown to

have a growth promoting effect on plants in some studies, particularly under the types of stress conditions which are sometimes associated with enhanced ethylene production such as drought or high salinity (Belimov et al., 2009 a, Belimov et al., 2009 b, Nadeem et al., 2010, Mayak et al., 2004a, Glick et al., 2007). It is assumed that bacterial regulation of ethylene accumulation influences plant growth because ethylene can directly negatively regulate plant growth at the cellular level (Dodd, 2005, He et al., 2009, Sobeih et al., 2004, Hussain et al., 1999, Wilkinson and Davies, 2010, Pierik et al., 2006). Studies here suggest an alternative or additional explanation for bacterial growth promotion. Bacterial growth promotion could occur through sensitizing stomata of aged or more mature leaves to any stress that produces ABA, such as drought, thus improving turgor for tissue expansion in more leaves per plant than in the non-inoculated plants. Tardieu et al. (2010) discuss the current understanding of the involvement of ABA in maintaining growth either through turgor maintenance or non-hydraulic growth regulation. Further work needs to be done to determine whether the growth promotion effect of bacteria occurs through turgor improvement in growing tissues or via a reduction in the more direct effect of ethylene to inhibit leaf cell extension rate, or both.

In order to determine whether ethylene-regulated leaf age effects on stomatal sensitivity to ABA occurred via age-dependent modulations of ethylene generation, or age-dependent modulations of target cell sensitivity, gas chromatography was used to measure ethylene production in different aged wheat leaves as described in chapter 2. However, ethylene generation from the leaves of this plant species and variety under these particular experimental conditions was too low to be detected in this system, except when leaves were pretreated with the ethylene precursor ACC (Figure 5.11). ACC content in plant tissues is related to the extent of ethylene production by that tissue as the rate limiting step of ethylene synthesis is ACC

synthesis (Wang et al., 2002, Guzman and Ecker, 1990, Kieber et al., 1993). Gas chromatography–mass spectrometry (GC-MS) was used here to measure ACC concentration, as a surrogate for ethylene production. As shown in Figure 5.10, there were no significant differences in the ACC concentration in leaves of different ages, both in the presence and absence of a pre-treatment with exogenous ACC. Nor were the amounts of ethylene generated by the ACC-pretreated leaves any different between age categories (Figure 5.11). Together, these data suggest that the lack of stomatal sensitivity to ABA in the older leaves could be due to an increase in stomatal sensitivity to ethylene, rather than to an age-related increase in ethylene production. As described above and together with reports of Desikan et al (2006) and Wilkinson & Davies (2010), ethylene closes stomata when ABA concentrations are relatively low, thus the concentration-dependent sensitivity of stomatal closure to ACC can be used as a surrogate for stomatal sensitivity to ACC or ethylene *per se* – whether the effect of ethylene causes stomatal closure or antagonizes ABA-induced stomatal opening.

To substantiate this hypothesis, three concentrations of ACC were applied to wheat leaves to induce ethylene production in plants. The data in Figure 5.8 show that, except for the lowest concentration, application of ACC could close stomata in the oldest leaves tested, but not in the two less mature leaves. As ACC can act as an independent signaling molecule in plant cells (Tsang et al., 2011), 1-MCP was used to investigate whether effects induced by ACC were indeed brought about through the ethylene that was generated as a consequence of its application. Stomata of less mature leaves, which had been most sensitive to ABA, showed only limited ACC-induced closure when pre-treated with 1-MCP, indicating that ethylene had indeed been the signal to which stomata in ACC-treated leaves were responding (Figure 5.9). These data, together with the findings that a given ACC application generates a similar amount of ethylene (and ACC) in leaves of all ages, indicates that

ethylene antagonizes ABA to a greater extent in more mature (aged) leaves is due to their greater sensitivity to ethylene, but not due to age-related differences in ethylene production (Figure 5.12). Such increased sensitivity to ethylene can explain the relative insensitivity of stomata to ABA and soil drying in older leaves, given that we know that ethylene antagonizes the stomatal response to ABA as shown in Figures 5.1, 5.2, 5.4 and 5.6 (and by Wilkinson and Davies (2010)). Antagonism between ABA and ethylene may be important to improve plant fitness to the environment in addition to matching carbon gain and water availability. For example, in sunflower, low nutrient availability stimulated ethylene production, thus increasing gs and transpiration thereby increasing soil nutrient abstraction rates (Nicotra and Davidson, 2010, Benlloch-Gonzalez et al., 2010). Both ethylene and ABA were involved in plant competition by regulating transpiration of competing plants (Vysotskaya et al., 2011). It will be interesting to explore how these hormones interact in different stresses such as heat, flooding, UVB where this interaction could allow plants to efficiently use water in a 'low-cost' way, thereby increasing fitness in a changing environment.

It has been shown that hydrogen peroxide synthesis is essential in the stomatal response to ethylene (Desikan et al., 2006). It is suggested that the enzyme nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase (AtrobohF) stimulated hydrogen peroxide production in response to ethylene in *Arabidopsis*, thus leading to stomatal closure. A further study of age-associated oxidative stress in plants suggested that chloroplastic antioxidant defenses levels decreased in aging plants (Munne-Bosch and Alegre, 2002). However, it is not clear whether increased stomatal sensitivity to ethylene with leaf aging is linked with age-associated oxidative stress in different leaves (Figure 5.12). It could be worthwhile to investigate this question in future work.

Interactions between ethylene and ABA occurred both at the level of synthesis where ABA restricted ethylene production to maintain shoot and root growth in low water relation conditions (Sharp, 2002) and in terms of cellular sensitivity where both hormones acted together to control seed germination (Ghassemian et al., 2000), but it would seem, in this case, that age-related changes in ethylene biology did not affect stomata through changes in ABA biosynthesis or catabolism, because there was no effect of leaf age on ABA concentration, in plants grown in either well-watered or drying soil (Figure 5.7). Therefore we propose that age-related changes in ABA sensitivity occur at the levels of an interaction between the guard cell ABA- and ethylene-signal transduction pathways, as previously proposed by Wilkinson and Davies (2010). In the future it will be important to use, molecular techniques to explore this interaction further. For example, the expression of ABA response genes under ethylene treatment by using RT-PCR.

Chapter 6 – General Discussion

Concerns about global food security are increasing. Increasing food production in a sustainable way is an essential component to balance the supply and demand of the food, particularly staple food crops such as wheat, rice and maize (Parry and Hawkesford, 2010). In the mid-1940s-1950s, Dr Norman Borlaug led a pilot program in Mexico, which developed high yielding semi-dwarf wheat varieties that resulted in a 6 fold increase of wheat yield between 1944 and 1963. This was known as the beginning of 'first green revolution'. To meet a target of 70% more food available by 2050 (FAO, November 2009) when facing factors such as land degradation, climate change which could cause yield reductions, a new green revolution is needed to increase crop yield. However, rather than a single 'magic bullet' such as semi-dwarf cereal crops used in the first green revolution, an integrated, interdisciplinary and sustainable approach is required to increase production per unit area and also optimize the resource use efficiency of crops (Parry and Hawkesford, 2010).

Over the past decades, many scientific efforts have aimed to manipulate 'above ground' traits (eg. Increasing crop harvest index with dwarf plant varieties), but genetic variation in root system properties has been largely neglected (Lynch, 2007). Root systems are currently attracting much scientific effort, as their manipulation could provide an approach to go beyond the first green revolution (Gewin, 2010). Lynch has highlighted the key role that may be played by the root system in reducing crop reliance on potentially expensive inputs such as chemical fertilizers and irrigation (Lynch, 2007). Wojciechowski et al. (2009) found the root length was decreased up to 33% in dwarf lines which contain *Rht-1Bc*, *Rht-D1c*, and *Rht12* dwarfing alleles in a common genetic background ('Mercia' and 'Maris Widgeon') when plants grew either

in pots with soil or in the field. Waines and Ehdaie (2007) also examined root characteristics in early green-revolution wheat lines and later-generation semi-dwarf lines and found that root biomass or size of modern cultivars is smaller in comparison with landraces. They suggested that this root trait may explain why dwarf genotypes carrying *Rht* dwarfing genes such as *Rht-B1c* have not been widely planted, particularly in dry environments.

As described in chapter 1, root architecture can have a crucial effect on nutrient and water uptake. Shallow roots usually allow plants to capture surface applied fertilizer such as immobile phosphate; while deeper roots may be required to explore deeper N reserves and access water in deeper soil layers. Ho et al. (2005) examined the correlations between shallow or deep roots and phosphorus and water acquisition in the greenhouse and field. Under combined water and phosphorus stress treatment, the best genotype was one with dimorphic root system (shallow and deep) in the greenhouse, while in the field genotypes with shallow roots grew best compared with deep-rooted or dimorphic root genotypes, indicating early vegetative growth is important for plants to cope with terminal drought environments. Watt's team is working with wheat lines which have deeper, faster-growing roots (Kirkegaard et al., 2007, Gewin, 2010). By crossing these lines with currently commonly used cultivars, 400 new wheat lines were developed and have being tested in the field in different regions of the world (Gewin, 2010).

Microbial manipulation is another approach to increase plant growth by regulating the growth and functioning of root system as discussed in chapter 1. Lopez-Bucio et al. (2007) observed that inoculation of *Arabidopsis* and bean with the PGPR strain *Bacillus megatenum* showed promoted shoot growth of bean and *Arabidopsis*, but inhibited primary root growth while increasing root hair length, and lateral root

number and growth of *Arabidopsis*. A recent report by Gutierrez-Luna (2010) showed that volatile organic compounds emitted by PGPR strains such as *Bacillus cereus*, and *B. simplex* can stimulate primary root growth and lateral root formation. PGPR strains which can produce indole-3-acetic acid (IAA) exhibited promotion effects on rooting in semi-hardwood and hardwood plants, indicating their potential usage in organic nursery material production as a replacement of synthetic auxin on rooting promotion (Erturk et al., 2010).

In addition to growth promotion effects on roots and shoots, the work presented in chapter 3 suggests another way that rhizobacteria could regulate plant development. *Variovorax paradoxus* 5C-2 which contains ACC deaminase not only stimulated floral transition in *Arabidopsis*, but also promoted *Arabidopsis* leaf growth. It is suggested that ethylene could be a major factor in the regulating plant growth and development in response to soil inoculation with this strain. Although rhizobacteria containing ACC deaminase have been shown to increase plant growth in different plants and under different conditions (Glick, 1995, Mayak et al., 2004a, Mayak et al., 2004b, Belimov et al., 2005, Belimov et al., 2009 b), very few studies reported the regulation of plant development by this group of rhizobacteria. Although Belimov et al (2009a) showed 5C-2 decreased time to flowering in potato, the mechanism causing this developmental regulation was not discussed. The work described in chapter 3 examined the potential role of ethylene in rhizobacteria-induced growth promotion and altered development of *Arabidopsis* by using ethylene mutants, measuring ACC concentrations in and ethylene production from *Arabidopsis* leaves, and determining transcription patterns of ethylene response genes. Taken together, these results suggest that soil inoculation with *V. paradoxus* 5C-2 promoted *Arabidopsis* growth and flowering via an ethylene-dependent pathway. Particularly, this work broadens the understanding that the regulation of ethylene status in plant by ACC deaminase

containing bacteria not only occurs locally in the root (Penrose et al., 2001), throughout young seedlings (Mayak et al., 2004a, Mayak et al., 2004b), or in the xylem sap (Belimov et al., 2009 b), but also in fully expanded mature leaves. This suggests ACC deaminase containing rhizobacteria could be used as a tool to mitigate short term ethylene over-production problems under serious stress conditions such as high temperature (Aloni et al., 1995, Djanaguiraman and Prasad, 2010) or ozone (Sinn et al., 2004, Vahala et al., 1998).

Effects of rhizobacteria 5C-2 on wheat were examined in chapters 4 and 5. Although 5C-2 stimulated floral transition and promoted vegetative growth in *Arabidopsis*, its effects on wheat were only observed in altered stomatal responses of aged leaves to soil drying and rehydration (chapter 5), but not in leaf growth of wheat seedlings at the 3rd leaf stage of plants grown in well watered or drying soil. Ethylene may have different effects on different plant species, particularly because of interactions between plant developmental stages and environment. Thus examining the effect of rhizobacterial inoculation on certain plant species under certain conditions is important before using bacteria in the field experiment. In the work here, only plant physiological and molecular responses were evaluated in response to rhizobacterial inoculation, while bacterial responses to plants were not examined. For example, it is not clear whether components of plant root exudates could regulate bacterial activity, population, or ACC deaminase activity in the rhizosphere under different environmental conditions. Investigating these variables in future experiments could benefit usage of ACC deaminase containing bacteria in different environmental conditions, especially in the field for the purpose of maintaining or improving crop yield.

The interaction between ABA and ethylene in regulating root and shoot growth has

been reported when plants face low shoot or root water potential (Sharp, 2002) or encounter compacted soil (Hussain et al., 2000). It is suggested that ABA maintains shoot and root growth by regulating ethylene production. Also, ABA and ethylene interact at the level of signal transduction in controlling seed germination (Ghassemian et al., 2000). However, only a few studies have explored ABA and ethylene interactions in regulating stomatal behavior. In ethylene treated *Arabidopsis* or ozone stressed *Leontodon hispidus* with enhanced ethylene production, stomata lost sensitivity to ABA (Tanaka et al., 2005, Wilkinson and Davies, 2009), as observed in older leaves by Atkinson et al. (1989) and the experiments reported in chapter 5. The work in chapter 5 provided insights into ABA and ethylene interactions in terms of stomatal regulation: Not only was the production of ABA and ethylene regulated to cause antagonistic effects on stomata as shown by Wilkinson and Davies (2009), but also in terms of changes in stomatal sensitivity to ABA and ethylene.

This is the first report that showed stomatal responses to ethylene are leaf age dependent. It suggests that when plants are setting seed, stomatal hyper-sensitivity to ethylene could lead to dramatically increased water loss when plants encounter ethylene stimulated by stresses such as high temperature (Djanaguiraman and Prasad, 2010, Hays et al., 2007) or ozone (Sinn et al., 2004). This problem could become worse if plants encounter multiple stresses such as drought with ozone (Wilkinson and Davies, 2009). Under these circumstances, rapid loss of water could cause plant water deficits, leading to a reduction of yield. Furthermore, if this leaf-age dependent sensitivity of ethylene is not only manifest as stomatal responses but also in the leaf cell, hyper-sensitivity to ethylene in aged leaves under stress conditions could cause more serious problems as ethylene is involved in inhibiting net photosynthesis (Kays and Pallas, 1980, Gunderson and Taylor, 1991, Pierik et al., 2006, Khan, 2004), modulating the timing of leaf senescence (Djanaguiraman and Prasad, 2010, Jing et al.,

2003), eventually leading to yield loss.

It has been reported in wheat that stress resistance or tolerance varied among genotypes and this can be associated with the levels of stress-induced ethylene production (Balota et al., 2004, Hays et al., 2007). Stress susceptible genotypes produced significantly more ethylene under optimum or stress conditions such as high temperature, or oxidative stress than stress resistance genotypes. It is suggested that ethylene synthesis can be increased (autostimulation) or decreased (autoinhibition) by ethylene (Arteca and Arteca, 1999, Yang and Hoffman, 1984, Chae and Kieber, 2005). However it is not clear whether ethylene sensitivity could be related to ethylene production under stress conditions and this could be used as an indicator for 'stress tolerance' across genotypes. Therefore, it will be worthwhile to examine relations between ethylene sensitivity and genotypic variation in stress. Probing the relationship between these two could provide a novel target for plant breeding programs for stress avoidance or for increased productivity.

Inoculation with rhizobacteria 5C-2 not only improved vegetative growth and stimulated floral transition in *Arabidopsis* as shown in chapter 3, but also increased root elongation at the early seedling stage in mustard (Belimov et al., 2005), and tomato (Belimov et al., 2007). Early root vigour is crucial for plants to take up water from deeper soil layers if plants face drought at a later growth stage (Palta and Watt, 2009). ACC deaminase-containing rhizobacteria such as 5C-2 could be used to improve root vigor. However, environmental factors could affect rhizobacterial populations and/or activity. For example, Martinez et al. (2011) reported that fertilizer such as N applied in the soil or soil pH regulated population size of the PGPR strains *Bacillus*, *Enterobacter*, *Pseudomonas* and *Serratia* and their trait activities such as IAA production or phosphorus liberation. In addition to soil characters,

competition within the microbial community of rhizosphere could strongly affect the survival or activity of PGPR strains, particularly the inoculated 'foreign' strain, since cooperation between plants and bacteria exhibits a high level of host specificity (Morrissey et al., 2004, Lugtenberg and Kamilova, 2009). These factors probably explain why many strains promote growth in laboratory experiments but do not always show consistent, effective performance in diverse field situations (Mark et al., 2006, Morrissey et al., 2004).

To obtain consistent and effective beneficial effects from bacteria on plant growth regardless of diverse environmental conditions, a genetic approach has been used to over-express the ACC deaminase gene from bacteria in tomato or canola using tandem constitutive cauliflower mosaic virus (CaMV) 35S promoters (Klee et al., 1991, Stearns et al., 2005) or specifically in the root of canola using a root specific promoter (Stearns et al., 2005). Reduction in ethylene synthesis was observed in leaves or fruit of transgenic tomato, in which 35S promoters was used to over-express *ACC deaminase* at whole plant levels, but no differences in apparent vegetative phenotypes were found between wild type and transgenic lines except significant delays in fruit ripening (Klee et al., 1991). Increased shoot and root growth was observed in transgenic canola either with 35S or root specific promoter lines at the seedling stage, but plant growth was enhanced in the transgenic line under the control of the root specific promoter when treated with nickel stress in soil. These studies suggest that tissue-specific over-expression of *ACC deaminase* may be a viable strategy to manipulate shoot ethylene production, especially since constitutive promoters could cause pleiotropic effects as basal levels of ethylene production are important for plant growth and development. As discussed, multiple stresses such as ozone and drought can potentially cause plant dehydration as increased transpiration during stress period due to the antagonism between ABA and ethylene (Wilkinson

and Davies, 2009). Therefore down-regulating ethylene production in the guard cell could be a potential approach to alter stomatal behavior under multiple stress conditions where both ABA and ethylene levels are increased at the same time. Recently, a strong stomatal specific promoter was isolated from *Arabidopsis* (Yang et al., 2008) and this offers a potential opportunity to specifically over-express bacterial ACC deaminase genes in the stomata in order to study ethylene function in the stomata. This could result in a new approach to help plants conserve water for later growth stages and thereby maintain crop yield under stress.

As discussed earlier, exploring and exploiting useful root traits could offer the opportunity of a second green revolution (Lynch, 2007). Using ACC deaminase containing rhizobacteria to regulate plant growth and development or stomatal responses under stress indicates strongly that ethylene production/sensitivity could be a target for breeding work to improve root performance. However, studies focused on ethylene effects on roots suggested that ethylene played a complicated role in regulating root growth and development, and the picture of ethylene function in roots is still not very clear. In *Arabidopsis*, application of ethylene gas or ACC reduced root elongation in a concentration-dependent way by decreasing root cell length (Dolan, 1997, Le et al., 2001). Sharp and co-workers suggest that ethylene can negatively regulate growth of primary maize roots at low water potential if ABA is deficient in those roots. Hussain et al (1999) showed that root-sourced ethylene inhibited leaf growth when plants encounter compacted soil. Gallie et al. (2009) reported that down-regulating the expression of ACC synthesis genes in maize decreased ethylene production, and increased growth of primary and seminal roots when plants were grown on filter paper. In addition to inhibition of primary root growth, ethylene is also involved in lateral root branching by inhibiting lateral root initiation and formation (Ivanchenko et al., 2008, Lewis et al., 2011), and cell division

modulation in the quiescent center in the stem cell niche (Ortega-Martinez et al., 2007).

Ethylene is involved in multiple net-works with other hormones including as ABA (Sharp, 2002, Ghassemian et al., 2000, Hussain et al., 2000), auxin (Tsuchisaka and Theologis, 2004, Stepanova et al., 2005, Stepanova et al., 2007, Ruzicka et al., 2007) and gibberellin (GA) (Achard et al., 2006) in regulating root growth and development. These hormonal interactions are regulated at multi-levels. For example, exogenous applications of either auxin or ethylene can alter the other's biosynthesis (Tsuchisaka and Theologis, 2004, Stepanova et al., 2005, Stepanova et al., 2008, Ruzicka et al., 2007); signalling or sensitivity (Stepanova et al., 2007); and ethylene can direct auxin transport in controlling cell expansion and lateral root formation (Lewis et al., 2011, Strader et al., 2010). In addition to net-works with other hormones, apoplastic alkalinization is also involved in ethylene-mediated regulation of root length or root cell length (Staal et al., 2011). Therefore, simply altering ethylene production or sensitivity could cause secondary effects since other signaling pathways such as the auxin pathway will be affected. For example, Strader et al. (2010) isolated an allele of the *eto1* ethylene overproducer and it restored auxin responsiveness to the auxin-resistant mutant *ibr5*.

Moreover, considering ethylene is involved in plant responses to different stresses (Wang et al., 2002), regulating ethylene biosynthesis or sensitivity could lead to different plant responses under different conditions. For example, Gallie et al. (2009) reported that a maize line with down-regulated expression of ACC synthesis genes displayed increased root growth in the filter paper system but decreased root biomass when plants grew in the soil. These complexities of ethylene functions in regulating plant growth and development, particularly in roots bring challenge in

using ethylene as a target in breeding or transgenic work. Therefore, more efforts need to be made to further our fundamental understanding of ethylene function as a plant growth regulator, especially in regulations of ethylene biosynthesis or sensitivity under different conditions in different regions of growing roots. Because high concentrations of exogenous ethylene or ACC and long incubation times (many hours to days) were used in earlier studies to uncover the effect of ethylene on root growth and development, and this probably is very different from what occurs in nature (Le et al., 2001).

In the field, plant growth and yield are affected by several factors such as soil properties, plant species and genotypes, and climate properties. Among these factors, the rhizosphere is crucial as the place where plants take up water and nutrients and interact with soil-borne microorganisms. Many factors influence the rhizosphere environment such as applications of fertilizer, irrigation, plant exudates, and soil-borne microorganisms. For example, applications of different types of fertilizer can affect rhizosphere pH (Ryan et al., 2009). Ammonium-based fertilizers can acidify the rhizosphere whereas nitrate-based fertilizers tend to alkalise the rhizosphere. Changes in pH can affect chemistry around the roots and microbial communities. Usage of organic manure also can increase soil microbial biomass and activity compared to those managed exclusively with mineral fertilizer, and also increase plant P uptake efficiency, thus improving yield (Simpson et al., 2011). Plants can modify the rhizosphere by releasing inorganic and organic substances from roots (Ryan et al., 2009). *AVP1 pyrophosphophatase* was over-expressed in *Arabidopsis* to increase H^+ efflux to the rhizosphere, leading to the acidification of the rhizosphere, which can increase nutrient acquisition by increasing Fe^{3+} and phosphorus availability (Yang et al., 2007). Inoculation of PGPR strains to the soil can promote plant growth directly by interacting with plants or indirectly by influencing rhizosphere microbial

communities. For example, introducing PGPR which produce antibiotics to the rhizosphere can suppress pathogen growth, thus improve health of plant roots (Ryan et al., 2009). Co-inoculation of ACC deaminase containing rhizobacteria and rhizobia can promote nodulation to a greater extent than rhizobial inoculation alone (Shaharoona et al., 2011, Zahir et al., 2011). The work presented here investigated effects of beneficial rhizobacteria containing ACC deaminase on plant growth, flowering time, and stomatal regulation which could increase plant water use efficiency under stress conditions. These strategies offer potential ways of using rhizobacteria to improve crop yield via rhizosphere engineering. Furthermore, mechanisms under lying rhizobacterial regulation of plant response(s) are explored, highlighting the role of ethylene in regulating plant responses to stress, thus opening a window for breeding and genetic modification work to target ethylene in their future work.

However, due to the complexity of rhizosphere chemistry and biology, predictabe rhizosphere engineering still remains a challenge. Further understanding of the complex chemical and biological interactions in the rhizosphere could help to develop more ecologically friendly agricultural practices. From the discussion above, it should be clear that no 'general' or 'universal' technique/method will secure food supply in the future. It seems clear that an interdisciplinary approach is needed to identify opportunities and develop innovative or improved techniques to improve crop yields in the future. In these new technologies or approaches, both genetic improvements of crops by genetic modification to introduce desirable traits or breeding new crop varieties and soil management practices in a more sustainable way are needed to contribute to food crop production. Considering regional diversity of global agriculture, there is no panacea for global food security and no techniques or technologies should be ruled out (Baulcombe, 2009).

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