Evaluating the role of *Bacillus amyloliquefaciens* QST 713 as a Plant Growth Promoting Rhizobacteria.

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Abstract

Due to an increasing need for sustainable crop management solutions, the use of biostimulants in agriculture is becoming much more widely researched. This study investigated the potential of *Bacillus amyloliquefaciens* strain QST 713 (Serenade ASO®) to promote growth of carrot and potato crops. Multiple controlled environment experiments were carried out using a variety of application methods, and different temperature regimes were also used to assess the effect of lower temperatures on bacterial growth both *in vitro* and *in vivo*. qPCR analysis was used to assess the ability of *B. amyloliquefaciens* to colonise potato and carrot root systems. After 28 days of potato growth, treatment with *B. amyloliquefaciens* resulted in a significant increase in root surface area (P= 0.045) and shoot height (P=0.047), as well as an increase in nutrient uptake. *Bacillus amyloliquefaciens* did not promote carrot growth at 20/15^{o}C or 20/10^{o}C after 21, 37 or 70 days of growth. However, when carrots were grown under a cooler temperature regime (15^{o}C/10^{o}C), *B. amyloliquefaciens* appeared to improve carrot root system development and nutrient uptake. Under the lower temperature regime, carrot root colonisation by *B. amyloliquefaciens* was superior to that observed under the warmer conditions (20^{o}C/10^{o}C). Enhanced root colonisation may have been due to the slower carrot growth rate and may explain the growth promotion effect observed under the cooler temperature regime. Results suggest *B. amyloliquefaciens* QST 713 could be an effective biostimulant for potato crops, but further research should investigate any yield benefits and the most efficient application method. Results also suggest that

further research should investigate the potential for *B. amyloliquefaciens* QST 713 to alleviate against cold stress in crops.

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List of Abbreviations

European Biostimulants Industry Council	EBIC
Plant growth promoting fungi	PGPF
Arbuscular mycorrhizal fungi	AMF
Plant growth promoting rhizobacteria	PGPR
Indole 3-acetic acid	IAA
Induced systemic resistance	ISR
Days after inoculation	dai
Days post inoculation	dpi
Colony forming units	CFU
Volatile organic compounds	VOCs
1- amino-cyclopropane-1-carboxylate	ACC
Ammonia	NH_3
Hydrogen Cyanide	HCN
Luria-Bertani	LB
Degrees Celsius	^oC
True leaf	TL
Days after sowing	das
Quantitative polymerase chain reaction	qPCR
Cycle threshold value	Cq
Melting temperature	Tm
Not detected	ND
Induced Coupled Plasma Mass Spectrometry	ICP-MS
Replicate	Rep
Hours	h

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Chapter 1 Introduction and Literature Review

1.1 Biostimulants

With the world population increasing rapidly and land for agricultural production a limited resource, global crop production is being forced to intensify (Gouda et al., 2018). However, to avoid a further decline in soil fertility and biodiversity, the use of more sustainable management practices will be crucial, and the adoption of biostimulants could be one way to achieve this (Gouda et al., 2018, Pérez-Montaño et al., 2014).

Plant biostimulants are used in agriculture worldwide to increase productivity with interest growing rapidly due to the sustainable nature of their use (Abbott et al., 2018, De Pascale et al., 2017). The increased reliance on agrochemicals over recent decades has caused great environmental concern and so the challenge of sustainably increasing global food production, whilst reducing environmental impacts, means that we must turn to more integrated approaches to crop management (Chandler et al., 2008). Components of integrated crop management (ICM) used alongside agrochemicals include biologicals, varietal resistance, cultural control methods and decision making tools (Chandler et al., 2008). Although biologicals are considered an important strategy in ICM, there are still relatively few biological products commercially available. This is thought to be due to regulatory difficulties and a limited understanding surrounding the mode of action and persistence of biologicals in the agricultural environment (Borriss, 2011, Chandler et al., 2008). With government restrictions on effective chemical control agents being implemented (Kinsella et al., 2009) and pathogens increasingly developing resistance to specific

chemical modes of action (Benítez et al., 2004, Fousia et al., 2015), the use of biologicals in integrated crop management is becoming much more widely researched (Fousia et al., 2015, Xu et al., 2011). It is evident that, before the application of biologicals becomes routine agricultural practice, extended research is needed into understanding the complex interaction between plants and biologicals (Borriss, 2011).

In 2014, the global biostimulant market was predicted to grow 12.5% annually from 2013 to 2018, resulting in a projected global market value of $3 billion by 2020 (Calvo et al., 2014, Colla and Rouphael, 2015). Biostimulants cover a wide range of substances and organisms including microbial inoculants, algae, seaweed extracts, amino acids, protein hydrolysates, humic acids, fluvic acids and even industrial waste. Reports of the potential plant growth promoting capabilities of these substances can be found throughout the scientific literature (Calvo et al., 2014, Polo and Mata, 2017).

1.1.1 Defining biostimulants

Due to the diverse range of plant biostimulants on the market, the sector sits in a difficult position regarding plant protection product policy in the EU (Matyjaszczyk, 2018). It is believed that biostimulants should be considered in a separate legislative group to chemical plant protection products as they often have multiple modes of action and so cannot be directly compared (Yakhin et al., 2016). However, some biostimulants are thought to possess plant protection benefits, microbial inoculants in particular, and this makes categorisation and legislation difficult (Yakhin et al.,

2016). The definition used by the European Biostimulants Industry Council (EBIC) is as follows, 'Plant biostimulants contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality' (EBIC, 2013). The EU however, is yet to come to an agreement on a specific definition of biostimulants (Matyjaszczyk, 2018).

1.1.2 Biostimulant functions

The scientific literature is full of recent studies and reviews of the use of plant biostimulants and their beneficial effects on crop growth, crop quality and tolerance to environmental stresses including nutrient deficiency, drought and salinity (De Pascale et al., 2017). In a broad sense, plant biostimulant products are thought to increase soil nutrient availability as well as plant uptake and assimilation of nutrients (De Pascale et al., 2017). However, as there are many different substances and micro-organisms which make up plant biostimulants, there are also many potential modes of action enabling this enhanced nutrient uptake and growth.

1.1.2.1 Biostimulant substances

Biostimulant substances include: protein hydrolysates which have been seen to cause beneficial hormonal effects and increase nutrient uptake in a range of crops (Colla et al., 2014) and seaweed extracts, some of which possess phytohormone-like properties, increasing plant nutrient uptake and stress tolerance (Battacharyya et al., 2015, Kaluzewicz et al., 2018). The exact modes of action of these biostimulants are still not fully understood; however, one theory is that they interact with plant

signalling, reducing the negative impact of stress on plants (Polo and Mata, 2017). If plants require less nutrients to overcome environmental stresses, more nutrients will be available for production and growth (Brown and Saa, 2015).

1.1.2.2 Biostimulant microorganisms

Microbial inoculants make up a large group of plant biostimulants and can be split up into two main groups: plant growth promoting fungi (PGPF), such as arbuscular mycorrhizal fungi (AMF) and *Trichoderma* species, and plant growth promoting rhizobacteria (PGPR) (Calvo et al., 2014, De Pascale et al., 2017, Rouphael et al., 2015, Rouphael et al., 2017).

PGPF such as *Trichoderma* species are commonly known for their bio-fungicidal properties (Benítez et al., 2004) . However, specific strains are also able to stimulate root growth and nutrient uptake, as well as increase drought and salinity stress tolerance in plants by altering root and shoot gene expression (López-Bucio et al., 2015). AMF contribute to plant growth promotion of most agricultural crops through a symbiotic relationship with crop roots (Calvo et al., 2014, Dorais and Alsanius, 2015). In return for a carbon source, AMF use their large network of hyphae to enable increased nutrient acquisition for the plant from beyond mineral depletion zones (Smith et al., 2011). AMF can cause phytohormone imbalances in crops, regulating and altering plant development and they can also increase drought and salinity tolerance (Rouphael et al., 2015).

PGPR are rhizosphere dwelling bacteria that colonise plant roots and consequently enhance growth (Kloepper et al., 1989). The rhizosphere is the area of soil that

surrounds plant roots. It was discovered by Hiltner in 1904 to contain a considerably larger proportion of bacteria than bulk soil (Hartmann et al., 2008). Plant roots secrete copious amounts of root exudates, which rhizobacteria use as nutrients, and this enables the rhizosphere to support much higher levels of microbial activity (Lugtenberg and Kamilova, 2009). The microbial composition of the rhizosphere is heavily dependent on the plant species present, the exudates released and the soil type (Berg and Smalla, 2009).

Among the bacteria considered as beneficial PGPR are both symbiotic bacteria and free living bacteria (Hayat et al., 2010). The form, function and uses of nitrogen fixing bacteria have been investigated heavily, for example *Rhizobium* species, which form symbiotic relationships with leguminous plants, dwelling in their root nodules and fixing nitrogen (Franche et al., 2009). Other nitrogen fixing bacteria are free living, such as species of the genera *Azospirillum*, *Enterobacter*, *Klebsiella* and *Pseudomonas* (Hayat et al., 2010). As well as nitrogen fixing PGPR, phosphate solubilising bacteria such as *Bacillus* and *Pseudomonas* species have been researched and used in agriculture for many years (Hayat et al., 2010). These beneficial PGPR contribute directly to soil nutrient availability, promoting plant growth.

1.2 Plant growth promoting rhizobacteria

1.2.1 Direct benefits

PGPR have both direct and indirect benefits to plant growth. Direct benefits occur due to a specific interaction between the plant and the microbe. These benefits have multiple modes of action and include: i) production of siderophores to facilitate the

uptake of nutrients such as iron, ii) production of phytohormones such as auxins to stimulate growth and alter root architecture, iii) production of enzymes and organic acids for solubilisation of minerals such as phosphate, iv) the asymbiotic fixation of nitrogen by species such as *Azospirillum* and v) the production of stimulatory volatile organic compounds (VOCs) (Bashan et al., 2004, Bhattacharyya and Jha, 2012, Borriss, 2016, Cabra Cendales et al., 2017, Calvo et al., 2014, de Freitas et al., 1997, Hayat et al., 2010, Khalid et al., 2004). PGPR increase root system biomass, increasing the area of the soil explored by plant roots (Calvo et al., 2014). This, in turn, combined with indole 3-acetic acid (IAA) production by PGPR can help to increase nutrient uptake and mitigate abiotic stress in crops, such as drought and salinity (Calvo et al., 2014, Hayat et al., 2010, Karlidag et al., 2013, Marulanda et al., 2009).

1.2.2 Indirect benefits

Indirect benefits to plant growth occur due to interactions between beneficial microbes and phytopathogenic microbes (Cabra Cendales et al., 2017). Increased plant growth can occur due to the suppression of pathogenic microbes and their effects by beneficial rhizobacteria (Lugtenberg and Kamilova, 2009). Effectively this is a method of biocontrol. A review paper by Whipps (2001) highlights the important modes of action used by PGPR in biocontrol, which include induction of plant resistance mechanisms, production of siderophores to compete for iron and competition between microbes for nutrients and colonisation sites. In addition to these, Borriss (2016) adds the ability of PGPR to suppress the negative effects that pathogenic microbes have on the native microbial diversity in the rhizosphere

(Erlacher et al., 2014). Other more aggressive modes of action include production of antimicrobial compounds which limit pathogenic growth, degradation of pathogen pathogenicity and germination factors, production of VOCs and the production of cell-wall degrading enzymes to break down pathogenic cells (Calvo et al., 2014, Whipps, 2001). The modes of action used by specific PGPR depend on the interaction between the plant, the microbe and other microbes in the rhizosphere (Whipps, 2001).

There are many examples of PGPR applied as soil inoculants (Mavrodi et al., 2012), seed treatments (Accinelli et al., 2018, Ji et al., 2006), or foliar sprays (Ji et al., 2006) that cause beneficial effects on crop growth, both directly and indirectly. Among the commercial biostimulants used worldwide are *Bacillus* species, which in 2007 took up approximately half of the commercially available bacterial biocontrol market (Ongena and Jacques, 2008). A review by Borriss (2016) summarises the various commercial strains of *Bacillus* species, which include *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens*, *B. mycoides* and *B. firmus*. This review will now focus on the PGPR species *B. subtilis* and *B. amyloliquefaciens* in detail. These species have been widely isolated and investigated for their plant growth promoting effects on crops for many years (Borriss, 2011, Chanway et al., 1988).

1.3 Bacillus species

Bacillus subtilis is considered a model Gram-positive bacterium, therefore much investigation has gone into the physiology and genetics of the species (Harwood and Cutting, 1990). *Bacillus subtilis* was the first Gram positive bacterium to have its genome sequenced (Kunst et al., 1997, Moszer et al., 1996). The species was chosen

not only because of the extensive knowledge of its properties but also due to its commercial uses (Kunst et al., 1997). *Bacillus subtilis* is a very diverse species which can be found in various environments such as in soil, bodies of water, the gastrointestinal tract of animals and on plant roots (Earl et al., 2008). *Bacillus subtilis* is well adapted for survival due to its ability to form dormant endospores which are persistent in adverse environments (Earl et al., 2008, Harwood and Cutting, 1990). When nutrients are limiting, sporulation occurs, and endospores are formed which are resistant to heat, radiation and desiccation (Hubbard et al., 2014). This allows *Bacillus* species to reside in the soil environment for very long periods of time without nutrient supply. Globally, *Bacillus* species are among the most widely used biologicals (Chagas et al., 2015) and this is thought to be due to the array of antimicrobial secondary metabolites that they produce, alongside their endospore forming properties which contribute to a long shelf life (Ongena and Jacques, 2008).

1.3.1 Biofilm production

PGPR such as *B. subtilis* colonise plant roots creating a biofilm and root exudates play an important role in enabling this (Lugtenberg and Kamilova, 2009). A process called chemotaxis means motile species such as *B. subtilis* move into the rhizosphere, drawn by the nutrient supply (Ongena and Jacques, 2008, Zheng and Sinclair, 1996). Once *Bacillus* is present next to the plant root, colonisation can occur. Biofilms are described as 'highly structured, surface associated communities' (Branda et al., 2001). Motile cells start biofilm construction by forming long chains of cells which align along the surface of the root; these produce aerial fruiting bodies, where highly resistant endospores are formed (Branda et al., 2001). This differentiation of cell

roles means that the *B. subtilis* biofilms can contribute to producing crucial antimicrobial secondary metabolites, as well as sporulate for persistence in the soil (Branda et al., 2001, Ongena and Jacques, 2008).

1.3.2 Biocontrol and antimicrobial secondary metabolite production

Studies have shown that the mode of action used by *B. subtilis* species to inhibit fungal pathogens directly is through formation of biofilms and production of antifungal lipopeptides (Bais et al., 2004). Within the many strains of *B. subtilis* is the genetic material to produce over 24 different antibiotics. On average, 4-5% of a *B. subtilis* genome is dedicated specifically to producing antibiotic secondary metabolites enabling important biocontrol mechanisms (Stein, 2005). The three important lipopeptides produced by *B. subtilis* species are surfactins, iturins and fengycins (Ongena and Jacques, 2008).

Production of surfactin is crucial to *B. subtilis* for the formation of biofilms, making it a vital lipopeptide in enabling root colonization (Bais et al., 2004). Surfactin is also crucial for colony spreading, swarming and induced systemic resistance (ISR) signalling (Ongena et al., 2007, Ongena and Jacques, 2008). Iturins, such as iturn A, exhibit strong antifungal properties (Aranda et al., 2005), as do fengycins, which also possess the ability to signal for ISR (Ongena et al., 2007).

In vivo, gnotobiotic experiments on root colonisation by *B. amyloliquefaciens* S499 have shown that surfactin makes up 90% of lipopeptide production during early biofilm formation and iturins and fengycins are produced in considerably lesser quantities later during colonisation (Debois et al., 2014). The quantity of lipopeptides

produced in the rhizosphere was also found to be much less than the quantity produced *in vitro* (Debois et al., 2014, Nihorimbere et al., 2012). Borriss (2016) therefore considers that although *Bacilli* PGPR have a large genetic capacity for production of antimicrobial compounds, the effect of these compounds in practice may not be as critical for biocontrol as first thought. Borriss (2016), in agreement with other studies (Choudhary and Johri, 2009, Fischer et al., 2013), goes on to highlight how inducing systemic resistance in plants could therefore be the main way in which *Bacillus* species suppress plant pathogens in the field.

Many different strains of *B. subtilis* have shown biological control potential on a range of important crop diseases, including foliar diseases (Rudrappa et al., 2010, Ryu et al., 2011) such as rusts (Li et al., 2013) and grey mould (Xu et al., 2016) and root diseases such as take all in wheat (Liu et al., 2010) and clubroot in oilseed rape (Lahlali et al., 2013). However, biofungicide efficacy in the field is often reduced due to insufficient coverage (Crane and Bergstrom, 2014), poor rain-fastness (Wei et al., 2016) and environmental factors such as temperature, sunlight, humidity and lack of soil moisture resulting in bacterial cell death (Peng et al., 2011, Reiss and Jørgensen, 2017). It has been suggested, however, that research into the environmental constraints of the bacteria, the number of doses, the dose rate and their use in combination with chemical fungicides and adjuvants could improve bacterial persistence and efficacy in the field (Reiss and Jørgensen, 2017, Wei et al., 2016). Pathogenic suppression is not only important for biocontrol purposes but also for providing indirect plant growth promotion effects.

1.3.3 Plant growth promotion

It is clear from the scientific literature that *Bacillus* species elicit a direct plant growth promotion effect. However, the exact mechanisms behind these beneficial effects are not completely understood and there are several potential explanations. A review by Berg (2009) describes how plant-microbe interactions not only promote plant health but also plant growth making them useful biopesticides, biofertilisers and phytostimulators. *Bacillus* strains promote growth directly through multiple modes of action, including production of phytohormone-like substances (Xu et al., 2016) and through increasing nutrient availability in the soil (Garcia-Lopez et al., 2018).

1.3.3.1 Phytohormone production

There is a considerable amount of work published on the growth promotion effect of various *B. subtilis* strains on tomato plants. Multiple papers report phytohormone production to be one of the beneficial contributing factors (Cabra Cendales et al., 2017, Mena-Violante and Olalde-Portugal, 2007).

Production of IAA, or auxin, is particularly common in *Bacillus* species (Chagas et al., 2015). IAA is an important plant signalling hormone; it regulates plant development, controls cell division and elongation and therefore stimulates plant growth (Teale et al., 2006). Higher IAA concentrations in plant roots have been associated with increased formation of lateral roots (Borriss, 2011). *Bacillus subtilis* strain GIBI 200 was found to produce high levels of IAA as well as possessing the ability to solubilise inorganic phosphates, both of which were thought to be responsible for the

increased tomato shoot length, root length and dry weight observed by Cabra Cendales et al. (2017). As well as IAA, *Bacillus* strains SG08-09 and SG09-12 were found to produce ammonia, siderophores, protease and cellulase *in vitro*, and also to solubilise phosphate (Xu et al., 2016). All or some of these properties were thought responsible for the significant increases in chlorophyll content, root and shoot length and dry and fresh weight of treated tomato plants in a greenhouse PGPR assay (Xu et al., 2016). Although IAA production is considered an important mode of action for PGPR, it was ruled out of being a contributing factor in the growth promotion of tobacco plants by *B. subtilis* and *B. amyloliquefaciens* strains in an experiment by Wang et al. (2009) due to the low concentrations produced by the strains *in vitro*. Idris et al. (2007), however, describe a positive relationship between the tryptophan-dependent synthesis of IAA and the ability of *B. amyloliquefaciens* FBZ42 to promote plant growth.

Many studies that consider phytohormone production to be responsible for the positive plant growth promotion effects of *Bacillus* species also consider the ability of *Bacillus* to solubilise phosphate an important factor (Borriss, 2011). The ability of *B. subtilis* to increase soil nutrient availability means that they are considered biofertilizers (Berg, 2009).

1.3.3.2 Biofertilisers

The majority of phosphate applied to agricultural soil as inorganic fertiliser is inaccessible to plants as phosphate ions are readily locked up as inorganic metal phosphates (such as Ca-phosphate or Fe-phosphate) in the soil matrix (Hinsinger, 2001). Equally, phosphate remains in organic form, for example as phytate which

cannot be hydrolysed by plants (Singh and Satyanarayana, 2011) and so low concentrations of phosphate ions are available in the soil solution for plant assimilation (García-López et al., 2016, Garcia-Lopez et al., 2018). Microbial inoculants with phosphate solubilising abilities, such as *Bacillus*, are therefore being investigated to improve the efficiency of P fertiliser in crop production.

Bacillus strains from the *Bacillus subtilis/amyloliquefaciens* group have the ability to solubilise inorganic phosphates such as Ca-phosphate and phosphate rock by acidification (Cabra Cendales et al., 2017). They also possess the ability to solubilise organic P by producing enzymes such as phytase, in turn increasing phosphate uptake by plants and promoting growth (Garcia-Lopez and Delgado, 2016, García-López et al., 2016, Idriss et al., 2002, Orhan et al., 2006, Singh and Satyanarayana, 2011).

Bacillus subtilis has the ability to obtain iron via a variety of methods: by producing extracellular siderophores which chelate iron and form soluble Fe-complexes (Xu et al., 2016), via assimilating Fe-complexes produced by other fungi and bacteria or by using citrate to chelate Fe(III) (Roy and Griffith, 2017). Secretion of siderophores and formation of soluble Fe-complexes make iron available for plant nutrient uptake and this can have a direct growth promotion effect (Xu et al., 2016). The production of siderophores was observed in *Bacillus* strain S32 in response to chromium toxicity in the soil and plant growth promotion effects were observed in *Brassica juncea* under both normal and abiotic stress conditions (Rajkumar et al., 2005). *Bacillus subtilis* commonly produces the catecholic siderophore bacillibactin (Temirov et al., 2003);

however, it has become apparent that some laboratory domesticated strains of *B. subtilis* (eg. JH642) can no longer produce the siderophore (Roy and Griffith, 2017).

Bacillus species have been seen to enhance the nutrient use efficiency of chemical fertilisers by supplementing their application (Adesemoye et al., 2009). *Bacillus* species used as biofertilisers can result in increased potassium availability in the soil, increased plant uptake of N, P and K and promotion of plant growth, all of which have been observed in eggplant (Han and Lee, 2005) and wheat crops (Sheng and He, 2006). The nutrient content of strawberry leaves and roots increased significantly when inoculated by *Bacillus* species under highly saline conditions and increases were observed in nitrogen, phosphate, potassium, calcium, magnesium, sulphur, manganese, copper and iron (Karlidag et al., 2013). Treatment of plant roots with *Bacillus* M3 has also been seen to stimulate raspberry growth, enhance the nutrient composition of the soil and plant leaves and increase yield (Orhan et al., 2006). Significant increases in N, P, Ca, Fe and Mn content of the leaves were observed, as well as significant increases in P, Fe and Zn levels in the soil. Soil N and K content however were not affected here (Orhan et al., 2006). It is evident that using *B. subtilis* as a biofertiliser can increase soil nutrient availability, directly boosting plant nutrient uptake and growth.

1.3.3.3 Enhancing root architecture

Multiple review papers describe the ability of PGPR species to enhance root architecture (Calvo et al., 2014, De Pascale et al., 2017). As well as significantly increasing the shoot height of cotton seedlings, root colonisation by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a has also been seen to have a beneficial effect on

root architecture, significantly increasing primary root length, lateral root number, lateral root length and root to shoot ratio. Inoculation with the combination of these strains also led to increased branching, thinner roots and an increased root surface area of the seedlings (Irizarry and White, 2017). Root colonisation by *B. amyloliquefaciens* was also found to significantly increase the root development of tomato and pepper plants resulting in an increase in total root surface area, total root length, projected area and the number of root tips (Kloepper et al., 2007). Kloepper et al. (2007) suggest this increase in root surface area and projected area will increase the area of soil explored by the roots and therefore have a positive effect on nutrient acquisition. In agreement, Dimkpa et al. (2009) suggest how observed increases in root surface area will be beneficial for nutrient and water uptake. The review by Dimkpa et al. (2009) highlights that multiple studies which find PGPR species to cause enhanced root growth and increased lateral root proliferation also observe production of IAA. However, Asari et al. (2017) conclude that the beneficial root architecture effects exhibited in Arabidopsis treated with *B. amyloliquefaciens* UCMB5113 were not necessarily just a result of auxin production. Asari et al. (2017) found *B. amyloliquefaciens* to stimulate lateral root elongation and root hair formation while decreasing the length of primary roots. This change in root architecture will therefore result in an increased exploration of the soil.

1.3.4 Persistence in the soil environment

Rhizosphere microorganisms will interact differently with plant roots depending on various biotic and abiotic factors (García-López et al., 2016). It is therefore important to consider growth conditions and soil properties when investigating the effect of

biostimulants. In order for microbial inoculants to be effective at promoting plant growth in the field it is imperative that we understand how these species behave in the soil environment and under changing environmental conditions (Asaka et al., 1996).

One of the most prevalent abiotic conditions affecting the growth and survival of bacteria is temperature (Budde et al., 2006) and so soil temperature should be an important consideration when applying bio-stimulants in field conditions. Previous studies suggest that *in vitro*, *B. subtilis* can grow within the temperature range of 11^{o}C to 53^{o}C, with optimum growth seen at 37^{o}C (Nichols et al., 1995). However, *Bacillus* strain EC15 was only found to grow in the narrower range of 15-32^{o}C *in vitro* (Marques et al., 2010). An *in vitro* study by Zheng and Sinclair (1996) found temperatures as low as 5^{o}C not to affect the chemotaxis response of *B. megaterium* to soybean root and seed exudates; however, optimum chemotaxis was achieved at 27^{o}C with Log CFU, motility and chemotaxis response reducing with lower temperature. In another *in vitro* study by Bae et al. (2013), temperatures in the range of 21-28^{o}C appeared to have no impact on the difference in ability between *B. amyloliquefaciens* GA4-4 and *B. subtilis* GA1-23 to inhibit mycelial growth; however, at 18^{o}C inhibition caused by *B. subtilis* GA1-23 was significantly reduced compared to that caused by *B. amyloliquefaciens* GA4-4. Previous work by Chow (2015, unpublished) on *B. amyloliquefaciens* strain QST 713 (synonymous with *Bacillus subtilis* QST 713) observed *in vitro* growth within 48 h at 25^{o}C; however, growth was not observed within the temperature range of 4-16^{o}C. When these cultures were then brought up to 25^{o}C growth was observed, suggesting that *B. amyloliquefaciens*

QST 713 spores will not germinate *in vitro* at temperatures lower than 16^{o}C. The effect of temperature on bacterial growth in the soil environment, however, is likely to differ, especially in view of the presence of plant roots, the production of root exudates and the chemical signals and nutrients that they exude, which favour biofilm production and bacterial persistence in the rhizosphere (Borriss, 2011).

Temperature increases metabolic cell activity, and this is thought to influence bacterial multiplication rate and root colonisation by *Bacillus* species (Kilian et al., 2000). Raaijmakers et al. (2009) describe how solar heating of the soil can increase the presence and activity of beneficial *Bacillus* species. In the UK, the soil temperature during the spring through to autumn growing season was observed by Kaczmarek et al. (2014) to be in the range of 9.5^{o}C to 22.5^{o}C at a depth of 20 cm. Budde et al. (2006) used 15^{o}C as a temperature to initiate a cold shock reaction in *B. subtilis in vitro,* which causes large adjustments in gene expression. This could suggest that lower UK soil temperatures, especially during the winter, could affect *Bacillus* growth and root colonisation. However, *B. subtilis* FZB24 was seen to colonise pea roots at temperatures as low as 10^{o}C, albeit with bacterial density decreasing by 2 orders of magnitude from 30^{o}C (Kilian et al., 2000). Schmidt et al. (2004) found that temperature regimes in the range of 3-35^{o}C appeared not to affect the population of *B. subtilis* MBI 600 found, mainly in spore form, colonising sugar beet seedlings. However, temperature was seen to affect the distribution of *B. subtilis* down the root system. Downwards colonisation and population density increased at temperatures in the range of 10-20^{o}C as opposed to lower and higher temperature ranges such as 3-12^{o}C, 15-25^{o}C and 25-35^{o}C. Pertot et al. (2013) found

temperatures in the range of 15-35°C to have no significant impact upon the root colonisation by *B. amyloliquefaciens* S499 on bean, tomato and zucchini roots, under sterilised conditions after 10 days. However, under hydroponic conditions after 4 weeks of growth, bacterial populations on roots were reduced from approximately 5 x 10^6 CFU g^{-1} of root at 35°C to 2.5 x 10^4 CFU g^{-1} of root at 15°C. Pertot et al. (2013) also suggest that temperature change has an impact on the physiology of *B. amyloliquefaciens* S499 cells and their ability to produce secondary metabolites.

A study by Asaka et al. (1996) compared the persistence of *B. subtilis* in the soil at 30°C and 15°C. After inoculation at 30°C, vegetative cell number dropped rapidly, and the number of spores increased with all viable cells surviving as spores two days after inoculation (dai), levelling out at 10^7 CFU g^{-1} dry soil. This final spore concentration was similar in the 15°C experiment, but the rate at which vegetative cells declined and sporulation occurred was much slower. Levelling out of spore numbers occurred at 10 dai rather than 2 dai. This study suggests that vegetative cells do not survive in the soil environment and survival occurs as spores, which prove very persistent. It may suggest that 15°C is a preferable temperature to 30°C for vegetative cells in the soil environment. This contradicts the optimal temperature of 37°C for *in vitro* growth (Nichols et al., 1995) but is in agreement with Tokuda et al. (1995), who in a similar experiment found that increasing the temperature of the soil increased the sporulation rate of *B. subtilis* NB22. Here *B. subtilis* populations also stabilised at 10^7 CFU g^{-1} of soil and spore persistence was observed for 50 days (Tokuda et al., 1995). In another similar study under sterile conditions at 18°C, *B. subtilis* MBI 600 was found to be in spore form 14 dai (Bennett et al., 2003). Bennett

et al. (2003) also observed that *B. subtilis* MBI 600 spores survived better in sterile soil than non-sterile soil. In the experiments discussed above no plant roots were present in the soil and so bacterial root colonisation could not occur, which could be a factor causing the rapid sporulation. However, a study by Elsas et al. (1986) found there was no difference in the rate of *Bacillus* recovery between fallow soil and the wheat rhizosphere.

Ben Abdallah et al. (2018) found that *B. amyloliquefaciens* colonised tomato roots at a stable rate up to 60 days at 25°C under sterilised conditions. However, when plants were inoculated with vegetative cells the number of bacteria recovered was 10^5 CFU g^{-1} compared to 10^7 CFU g^{-1} when spores were used. Elsas et al. (1986) showed that although *Bacillus* was found to reside in the soil mainly in spore form, at 7 dai and 30 dai *Bacillus* was mainly in its vegetative state, suggesting that biofilm production and cell differentiation plays an important role in *Bacillus* persistence in the soil (Branda et al., 2001). More recently, Chowdhury et al. (2013) found *B. amyloliquefaciens* capable of colonising lettuce roots in the field with *Bacillus* CFU seen to reduce from 7.45 Log_{10}CFU g^{-1} at planting to 6.61 Log_{10}CFU g^{-1} after five weeks. In a greenhouse experiment, Chowdhury et al. (2013) observed spore density in the rhizosphere to decline over a four week period although vegetative cell density remained stable, albeit at lower levels.

Another important factor affecting root colonisation is soil type. A study by Garcia-Lopez et al. (2018) revealed that not only did soil type cause a significant effect on the CFU count of *B. amyloliquefaciens* QST 713 found in the soil at harvest but also on the beneficial increase in dry matter and P concentration of cucumber roots.

Bacillus amyloliquefaciens CFU were found to correlate positively with the clay content and soil organic matter of the soil and negatively with the electrical conductivity and the β-glucosidase activity of the soil. β-glucosidase activity acts as an indicator of the microbial activity in the soil. As β-glucosidase activity increased the CFU count of *B. amyloliquefaciens* QST 713 in the soil decreased, indicating that the quantity of *B. amyloliquefaciens* in the soil is affected by competition with other microorganisms. However, in this experiment CFU count did not significantly influence the observed plant growth promotion benefits.

The studies described here show how both temperature and soil type affect the growth of *Bacillus* species in the soil. Although studies show that *Bacillus* species do persist in the soil, there is a current lack of understanding as to whether *Bacillus* strains can germinate and colonise roots in colder soil environments, such as those in the UK.

1.4 *Bacillus amyloliquefaciens* QST 713

Bacillus amyloliquefaciens strain QST 713 (synonymous with *Bacillus subtilis* QST 713) was discovered by AgraQuest, Inc., USA and subsequently commercialised into the biofungicide Serenade® (Edgecomb and Manker, 2006). The strain was first approved for registration as a foliar fungicide in Chile in 1999. Following this, approval was granted in the United States in 2000 and in many other countries across the globe in succeeding years, including France, Germany, Australia and the UK (Edgecomb and Manker, 2006). In 2012, AgraQuest, Inc., USA was bought by Bayer CropScience, who continue to sell QST 713 and its secondary metabolites as Serenade® ASO. Although *B. amyloliquefaciens* QST 713 is predominantly sold as a

biofungicide to control a range of plant diseases including downy mildew, fire blight and tomato leaf spot (Borriss, 2011), this thesis will collate the evidence from the scientific literature regarding the role of *B. amyloliquefaciens* QST 713 as a biostimulant.

1.4.1 Classification

Bacillus strain QST 713 was previously classified as *B. subtilis* QST 713. However, due to improved genetic and sequencing technologies the strain has been reclassified to *B. amyloliquefaciens* QST 713 (Mendis et al., 2018). The following section describes and explains this reclassification.

There are four species which comprise the *Bacillus subtilis* group, specifically, *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. pumilus*. All the members of the *B. subtilis* species group share a core genome consisting of 2139 genes (meaning each gene is > 50% identical) (Chen et al., 2009). The majority of genes found in *B. amyloliquefaciens* FZB42, of the subsp. *plantarum* (Qiao et al., 2014) are also found in *B. subtilis* 168, meaning the species are very closely related (Chen et al., 2009). *Bacillus subtilis* 168 is a domesticated, model organism which was the first Gram positive bacterium to have its genome sequenced (Earl et al., 2008). Unlike *B. subtilis* 168, however, *B. amyloliquefaciens* FZB42 is plant-associated, meaning it can colonise plant roots effectively (Qiao et al., 2014). Previously these species would have been classified by morphological, physiological and biochemical traits, but with modern molecular technologies such as sequencing, these species can now be classified by their genetics (Mendis et al., 2018).

Bacillus amyloliquefaciens FZB42, which until 1987 was considered a subspecies of *B. subtilis* (Priest et al., 1987), contains gene clusters responsible for the production of important secondary metabolites called polyketides, including bacillaene, difficidin, oxydiffidicin and macrolactin. The ability of specific strains of *Bacillus* species to produce these polyketides has become important in distinguishing between strains in the *B. subtilis* group.

The differences in the production of these secondary metabolites by *B. amyloliquefaciens* and *B. subtilis* were investigated by Chen et al. (2009). By looking at the gene clusters responsible for producing polyketides and phylogenetic trees based on the alignment of the *cheA* (chemotaxis A) and *gyrA* (gyrase subunit A) nucleotide sequences, the study uncovered that *B. subtilis* strain QST 713 is part of the *B. amyloliquefaciens* group rather than *B. subtilis*. Figure 1-1 is taken from Chen et al. (2009) and shows the differences between the two phylogenetic groups and the gene clusters responsible for polyketide production in the various strains of *Bacillus*. It is clear here that *B. subtilis* QST 713 is more genetically comparable to *B. amyloliquefaciens* FBZ42 than *B. subtilis* 168. A recent publication by Mendis et al. (2018) confirms this, stating that *B. subtilis* QST 713 has now been taxonomically classified as *B. amyloliquefaciens* QST 713. By 2020 it is expected that across all countries Bayer CropScience registrations for strain QST 713 will be reregistered as *B. amyloliquefaciens*. Therefore, in order to keep in line with current taxonomic classification the formerly named *B. subtilis* QST 713 will be referred to as *B. amyloliquefaciens* QST 713 in this thesis. The strain QST 713 is also referred to as *B. velezensis* QST 713 in other studies (Meng et al., 2016). Borriss (2011) describes how

Bacillus strains GB03, QST 713, MBI 600, GA1, A1/3 and FBZ42 are all genetically very closely related.

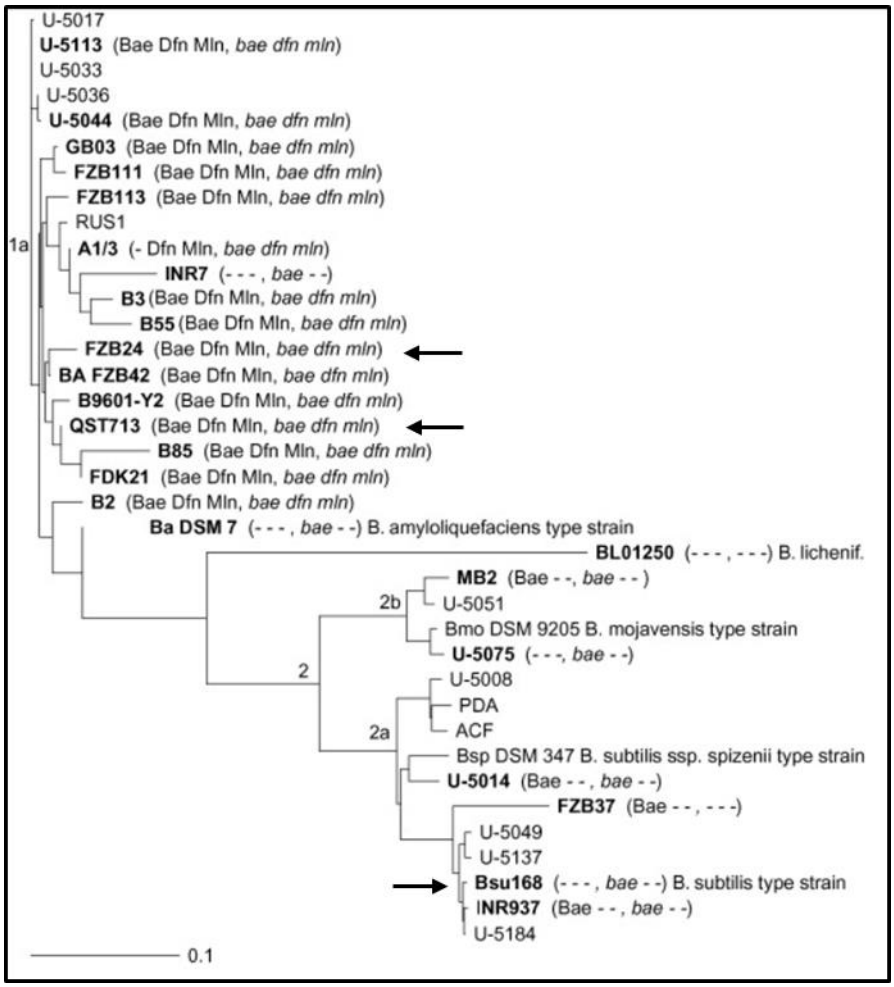

Figure 1-1: Phylogenetic tree from Chen et al. (2009) based on the alignment of partial cheA *nucleotide sequences to demonstrate the gene clusters (Bae Dfn Mln,* bae dfn mln*) present for antibiotic production in strain QST 713. Numbers 1a to 2b indicate different branches of the phylogenetic tree. Strain* QST 713 *possess the same gene clusters as strain* FZB42 *(species indicated with arrows) in branch 1a, whereas* B. subtilis 168 *(also indicated with an arrow) in branch 2a does not possess these gene clusters.*

1.4.2 Plant growth promotion potential

Much of the scientific literature surrounding the growth promotion effects of *B. amyloliquefaciens* QST 713 focusses on the effects observed when plants are initially compromised by disease, in effect the biocontrol efficacy of the strain. There are, however, some studies on the direct growth promotion effects of strain QST 713, along with similar strains, looking at the possible mechanisms responsible.

The ability of *Bacillus* species to increase soil nutrient availability is considered an important mode of action for their role in plant growth promotion. García-López et al. (2016) found inoculation by *B. amyloliquefaciens* QST 713 to significantly increase cucumber root dry matter yield, as well as increase phosphate content in roots and shoots, increasing total P uptake by 40%. A recent study by García-López et al. (2018) also revealed QST 713 to be an efficient mobiliser of soil phosphate, increasing P availability and uptake in wheat under controlled environment conditions. Dry matter yield of treated wheat crops was significantly increased, thought partly due to more available soil P. Both these studies suggest *B. amyloliquefaciens* QST 713 treatment increases root proliferation and phosphate uptake.

García-López et al. (2018) formulated a model to estimate the ratio of P uptake in inoculated plants compared to control plants which was found to be a function of several soil factors: phytase-hydrolysable P, initial soil Olsen P, calcium carbonate content, the ratio of Fe in poorly crystallized oxides to Fe in crystalline oxides, and citrate soluble P ($R^2 = 0.92$). Olsen P is an estimation of the plant available P in soils and is determined by extraction with sodium bicarbonate (Olsen et al., 1954). This

model suggests that the form of phosphate in the soil has a strong impact on the P solubilisation potential of QST 713. Plant P uptake increases with higher levels of phytase-hydrolysable P in the soil and with reduced levels of the other forms of P. Solubilisation of organic P forms by QST 713 is therefore thought to be an important contributor to increasing plant P uptake. Similarly, Ramirez and Kloepper (2010) suggest that production of phytase by *B. amyloliquefaciens* FBZ45, a similar strain, to solubilise organic phytate in the soil is the major mechanism resulting in plant growth promotion. The study found that growth promotion of Chinese cabbage by FBZ45 was only seen when phytate was present and the soil was of a low soluble phosphate concentration. It is suggested that the presence of organic phytate in the soil could be a limiting factor of P-solubilisation by certain *Bacillus* PGPR. However, in the study discussed previously by Garcia-Lopez et al. (2018), although the density of *B. amyloliquefaciens* CFU positively affected the P solubilisation in the rhizosphere, it did not correlate with increased P uptake by the plant and so it is suggested that P solubilisation may not be the only factor resulting in increased plant P uptake (García-López et al., 2016, Garcia-Lopez et al., 2018). Garcia-Lopez et al. (2018) also conclude that the effect of *B. amyloliquefaciens* QST 713 could be due to acidification of the rhizosphere causing inorganic metal phosphates to dissolve. Although the model described above shows that a decrease in citrate soluble P (eg. Ca-phosphates) correlates with increased P uptake, this is thought to be due to a preferred solubilisation of organic P by the bacteria. As rhizosphere acidification correlated negatively with both Olsen P and levels of organic P, it is thought both these concentrations in the soil will be responsible for the mode of action used by the bacteria to solubilise phosphate.

Another important PGPR mode of action to be considered is the effect that secondary metabolites produced by QST 713 have on plant chemical signalling. Both *B. amyloliquefaciens* strains FZB42 and BAC03 have been known to promote plant growth and yield under pathogenic pressure through effective reduction in disease severity via production of antimicrobial secondary metabolites (Meng et al., 2013); however, Meng et al. (2016) undertook a study to further understand the potential of multiple *Bacillus* strains including BAC03, FZB42 and QST 713 to promote plant growth in the absence of disease. The study looked at multiple horticultural crops, including carrot, potato, radish, beet, cucumber and tomato. Applied at 10^5 CFU/cm^3, strain BAC03 exhibited growth promotion in shoot height, leaf fresh weight and leaf dry weight in all crops and significant increases in fresh and dry root weight were seen in the root crops carrot, beet, radish and turnip. Increases in radish dry weights were observed and positively correlated with increased concentration and application number of the inoculant. Strain QST 713, applied at 10^5 CFU cm^{-3} at 10 days post inoculation (dpi), also significantly increased radish leaf and root, fresh and dry weights. Strain QST 713 was capable of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase production, IAA production and ammonia (NH_3) production, although levels were significantly lower than by strains BAC03 and FZB42 (Meng et al., 2016). Interestingly, siderophore production was not detected. However, Manker (2012) claims *B. amyloliquefaciens* QST 713 produces siderophores for increased iron uptake. Non-detection could be due to the method used by Meng et al. (2016), which is described by Schwyn and Neilands (1987). However, multiple studies cite this method for successful detection of siderophore production in *Bacillus* species (Agarwal et al., 2017, Liu et al., 2017). The production of the compounds discussed

above could all be associated with the resulting plant growth promotion effects observed in these crops.

Production of IAA, hydrogen cyanide (HCN), NH_3, siderophores, and ACC deaminase *in vitro* has also been seen in *Bacillus* strain EC15 (Marques et al., 2010). The same isolate was seen *in vivo* to significantly increase shoot and root elongation, shoot biomass, shoot P accumulation and root N accumulation in *Zea mays*. Significant positive correlations were observed between both IAA and NH_3 production and all the plant traits discussed above, and between siderophore production and N accumulation and biomass production. In agreement, Idris et al. (2007) found members of the *B. subtilis/amyloliquefaciens* group able to produce IAA both *in vitro*, on maize seedlings when grown at 22^{o}C under low oxygen (Idris et al., 2004), and *in vivo*, where IAA production exerted potent plant growth promotion effects on duckweed.

The phytohormone ethylene is known to inhibit seedling root growth. However, ACC deaminase production causes the breakdown of ACC, the precursor for ethylene, reducing ethylene production by the plant and allowing for increased root growth (Penrose and Glick, 2003). ACC deaminase production has been seen in multiple *Bacillus* strains, including QST 713 (Meng et al., 2016), which could also be contributing to plant growth promotion effects.

The *Bacillus* strains QST 713, FZB42, and BAC03 all exert growth promotion effects on crop plants. However, interestingly, they were observed to significantly inhibit radish seedling germination and growth, *in vitro*, by 80-90% and 40-50% respectively (Meng et al., 2016). Inhibition was thought to be due to the release of volatile

organic compunds (VOC's), specifically putative 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, which are thought to be responsible for some of the biocontrol properties of these species. Although seedling growth and germination appeared to be inhibited by these VOCs, Meng et al. (2016) concluded that this may have been due to the high concentrations on one small seed. This is in agreement with Ryu et al. (2005), who studied the effect of *Bacillus* IN937a and GB03 metabolites on *Arabidopsis thaliana* both *in vitro* and *in vivo*. *In vivo*, growth promotion was observed, and *in vitro* results indicated a growth promotion effect when the bacteria were placed 6 cm from the seed, whereas at a 2 cm distance the same metabolites caused seedling death. Ryu et al. (2005) concluded this result was due to differing concentrations and compositions of inhibitory compounds, therefore it is thought that if concentrations are diluted in the soil environment, inhibitory compounds such as VOCs will in fact benefit growth as the plants mature (Meng et al., 2016). The beneficial effect of VOCs is also backed up by Ryu et al. (2003), who found *Bacillus* strains IN937a and GB03 significantly increased the growth of *A. thaliana* seedlings *in vitro*, while consistently releasing the VOCs 3-hydroxy-2-butanone and 2,3-butanediol. In agreement, Borriss (2011) describes how mutants of *B. amyloliquefaciens* FZB42 inhibited from producing volatiles were found to lose their plant growth promotion ability. These studies support the theory that VOCs play an important role for chemical signal triggering and growth promotion by *Bacillus* species.

As well as significantly reducing the disease severity of scab (*Streptomyces scabies*) on potatoes under field conditions, *B. amyloliquefaciens* BACO3 also caused a

significant increase in yield, increasing tuber weight up to 33% (Meng et al., 2013). In the same study, under greenhouse conditions, treatment with BACO3 was observed to increase the fresh weight of radish leaves and roots by 126% and 166% respectively.

There is increasing interest in the agrochemical sector in incorporating microbial inoculants into chemical seed coatings to reduce environmental impacts. However, a long shelf life and sustained viability are crucial (Accinelli et al., 2018). When *B. amyloliquefaciens* QST 713 was applied to canola and maize in a bioplastic matrix seed dressing, Accinelli et al. (2018) found that *B. amyloliquefaciens* promoted seedling growth, significantly increasing both shoot and root length in maize by 18% and 21.4% respectively.

It has been suggested that different methods of growth promotion occur *in vivo* and *in vitro*, as strains of *Bacillus* which promoted growth significantly *in vitro* were only moderately effective *in vivo* (Ryu et al., 2005). However, this may also be due to changing environmental conditions and survival of the bacterial cells *in vivo* (Reiss and Jørgensen, 2017).

Although there is an abundance of studies to show the plant growth promotion effects of many other PGPR species (Dimkpa et al., 2009), studies on the plant growth promoting effects of *B. amyloliquefaciens* strain QST 713 where plants are not compromised by disease are less common.

1.4.3 Biofungicide potential

Bacillus amyloliquefaciens QST 713 has been found to control plant diseases of multiple agricultural crops both *in vivo* and *in vitro* (Hibar et al., 2006) resulting in indirect plant growth promotion. *Bacillus amyloliquefaciens* QST 713 has been reported capable of controlling foliar (Abbasi and Weselowski, 2015, Fousia et al., 2015, Reiss and Jørgensen, 2017), soilborne (Lahlali et al., 2013, Zeng et al., 2012b) and post-harvest diseases (Punja et al., 2016, Restuccia et al., 2006) in a range of economically important crops. The use of *B. amyloliquefaciens* QST 713 as a seed treatment has also been investigated (Accinelli et al., 2018, Özer and Bayraktar, 2014). Although many studies report the successful biocontrol effects of *B. amyloliquefaciens* QST 713, often they are considerably less effective under field conditions (Peng et al., 2011, Reiss and Jørgensen, 2017, Zeng et al., 2012a).

1.5 Quantifying bacterial root colonisation

Successful root colonisation by beneficial bacteria is vital for resulting plant growth promotion effects (Lugtenberg and Kamilova, 2009) and so it is important that bacterial root colonisation is quantified when assessing the effects of PGPR on crop growth (Kloepper and Beauchamp, 1992).

In 1996, a gnotobiotic method for detecting bacterial rhizosphere colonisation was developed which involved inoculating sterile seeds with the beneficial bacteria and planting them in sterile quartz sand in a sterile glass tube (Simons et al., 1996). This allowed for dilution plating techniques to accurately determine bacterial CFU counts. More recently, Ben Abdallah et al. (2018) also used sterile soil and determined the

colonisation ability of *B. amyloliquefaciens* on tomato roots by vortexing root samples in Tween 20 and dilution plating onto Luria-Bertani (LB) media. These methods however are not as useful for experimentation using un-sterilised soil media and results are not comparable to field situations due to the lack of native soil microbial activity (Kloepper and Beauchamp, 1992). Multiple scientists quantifying *B. subtilis* CFU in un-sterile soil have used a method by Tuitert et al. (1998) involving rhizospheric soil extraction by sodium pyrophosphate and isolation of *B. subtilis* on nutrient agar following heating at 80^{o}C for 10 min (Elsas et al., 1986, García-López et al., 2016, Garcia-Lopez et al., 2018). However, prior knowledge of specific colony morphogenesis of *B. subtilis* as opposed to other *Bacillus* species is required. To avoid determining CFU by colony morphology, Asaka et al. (1996) used antibiotic resistance and dilution plating; and more recently studies have used dilution plating onto semi-selective tryptic soy agar (TSA) amended with cycloheximide to determine *B. subtilis* CFU (Bennett et al., 2003, Chowdhury et al., 2013).

Other scientists have used scanning electron microscopy and confocal scanning laser microscopy (Bais et al., 2004, Fan et al., 2012, Rudrappa et al., 2010) to evaluate root colonisation by *Bacillus* species. However, more recently scientists have used quantitative polymerase chain reaction (qPCR) technologies to detect and more accurately quantify PGPR DNA in the rhizosphere and on the root surface (Couillerot et al., 2010, Wang et al., 2017). To test whether *B. amyloliquefaciens* was capable of colonising canola roots, Lahlali et al. (2013) used qPCR to detect DNA on the root system, finding that *B. amyloliquefaciens* was able to colonise canola roots effectively within seven days, and the amount of *B. amyloliquefaciens* DNA in

inoculated canola roots increased from seven dpi to 14 dpi showing the ability for continued growth. Accinelli et al. (2018) used qPCR to detect the presence and the increasing abundance of *B. amyloliquefaciens* in the soil after using *B. amyloliquefaciens* QST 713 as a seed treatment.

1.6 Factors affecting PGPR efficacy

A large number of factors affect plant-microbe interactions, root colonisation and therefore biostimulant efficacy. These factors include temperature, water availability and solute concentration, as well as other abiotic and biotic factors (Cray et al., 2013) which cannot be controlled in the field meaning that efficacy in the field is often reduced and difficult to predict (Cray et al., 2015). Borriss (2016) therefore suggests that research into PGPR must be carried out *in vivo* on plants, or at least where biofilm production is possible to account for environmental differences. Factors which need to be considered when a microbial inoculant is brought onto the market include the optimum dosage of the inoculant, the way in which it is formulated (Lahlali et al., 2013), the application technique and the timing of application (Borriss, 2016, Meng et al., 2016). Shelf life and the response of the inoculant to different environmental conditions, including moisture, temperature, and soil type, also need to be investigated thoroughly (Bashan et al., 2014, Berg and Smalla, 2009, De Pascale et al., 2017). A recent review by Gouda et al. (2018) suggests that there is a gap between how PGPR are used currently, and how they can be used for more reasons on a wider scale to improve the sustainability of agriculture. Areas where PGPR are less successful in the field need to be investigated

by multidisciplinary approaches in order to improve efficiency and agricultural productivity (Gouda et al., 2018).

1.7 Demand for biostimulants

Organic horticulture was said to double globally between the years 2003 and 2013 due to increasing consumer demand for organic fruit and vegetables (Dorais and Alsanius, 2015). This dramatic increase provides a growing gap in the market for biostimulant products to aid a drive towards not only increasing organic yields, but also providing alternative tools for more sustainable conventional systems (Hayat et al., 2010). In addition, a reduction in the number of effective pesticides in the conventional sector and an increasing customer demand for sustainable, safer, residue-free food (Borriss, 2011, Gerbore et al., 2014, Ravensberg, 2015) means there is space for PGPR use in both conventional and organic farming systems.

1.8 Aims

This study had two main aims:

1) To investigate any growth promotion effects that *B. amyloliquefaciens* QST 713 has on the high value UK horticultural crops carrots and potatoes when they are uncompromised by disease. Experiments aimed to explore potential modes of action responsible for any plant growth promotion observed *in vivo*, such as changes in root architecture and nutrient uptake.
2) To understand some of the environmental limitations of *B. amyloliquefaciens* QST 713 growth in the soil environment, more specifically the effect of lower UK soil temperatures on *B. amyloliquefaciens* QST 713 root colonisation. This

study also aimed to understand how the application method of *B. amyloliquefaciens* QST 713 can deliver the best results for any potential plant growth promotion effects.

1.9 Hypotheses

- Root colonisation by *B. amyloliquefaciens* QST 713 directly stimulates growth of potato and carrot roots leading to increased nutrient uptake

- Lower soil temperatures affect the root colonisation and persistence of *B. amyloliquefaciens* QST 713

- The method used to apply *B. amyloliquefaciens* QST 713 affects its ability to colonise roots and stimulate growth

Further to these hypotheses, more specific sub-hypotheses are given for each of the experiments at the beginning of chapters three to five.

Chapter 2 General Materials and Methods

2.1 Experimental set-up

2.1.1 Preparation of columns and soil media for controlled environment experiments

PVC columns of varying sizes were used to grow both carrots and potatoes over a range of time periods. Potatoes (Exp.1) were grown in columns with an average diameter of 14.4 cm and height 1 m. Carrots were grown for 21 days (Exp. 2a), 35 days (Exp. 2b) and 70 days (Exp. 3b) in the column sizes 7.5 cm (diameter) x 25 cm (height), 14.4 cm x 50 cm and 14.4 cm x 1 m, respectively. A sterilised Kettering loam and sharp sand (55%) mix was used as the growth medium for all *in vivo* experiments. After filling the columns with soil, they were saturated and left to drain for two days, prior to being sown. This allowed them to reach a water content representative of the water holding capacity of the soil under these conditions.

2.1.2 *Bacillus amyloliquefaciens* QST 713 treatment

Bacillus amyloliquefaciens QST 713 treatment was applied to columns as Serenade ASO® (Bayer Crop Science Ltd., *Bacillus amyloliquefaciens* QST 713) at differing rates, using various application methods, dependent on the experiment. Serenade ASO® was applied at a rate of 5 L ha^{-1} in 100 L of water for potatoes (Exp.1) and 10 L ha^{-1} in 200 L of water for carrots (Exp. 2a, 2b & 3b). Serenade ASO® suspension was made up by adding 1 mL of concentrated Serenade ASO® to 20 mL of water and mixing thoroughly. The dose of Serenade ASO® suspension applied per column was calculated using the area of the soil surface and is detailed in each chapter. The

concentration of *Bacillus amyloliquefaciens* CFU in the Serenade ASO® suspension was determined by dilution plating to be 4.9 x10^8 CFU mL^{-1}.

2.2 Growth parameter assessment

2.2.1 Harvest measurements

At the end of the growing period, columns were split open to reveal the soil and root matrix. Shoot height was measured from the soil surface to tallest part of the shoot and then above ground plant material was oven dried to calculate shoot dry weight. Prior to drying, carrot shoots (Exp. 2b & 3b) were passed over a LI-3100 area meter (LI-COR Inc, Lincoln, Nebraska, USA) to determine leaf area. Rooting depth was measured between the soil surface and the deepest root tip. Following this, soil layers (including roots) were sectioned into different depths dependent on the experiment and stored at 4°C until processing.

2.2.2 Root quantification

Roots were extracted from the sectioned volumes of soil in one of two ways: hand washing, which involved washing the soil through a 1 mm sieve and extracting the roots, or using the Delta-T RWC-UM-2 Root Washer, which separates the roots and organic matter from the bulk soil. Following use of the root washer, any remaining organic matter was removed by hand. The method used in each experiment is detailed within each chapter. Roots from each section were scanned on a flatbed scanner (EPSON expression, 11000XL Pro, Japan) and analysed using WinRHIZO® software (Regent Instruments Inc., Québec, Canada). This allowed calculation of total root length, total root surface area and the average root diameter for each column section. Dividing the total root length by the volume of soil enabled calculation of

root length density (cm cm^{-3}). Following scanning, roots were oven dried to calculate below ground dry weight.

2.3 Nutrient Analysis

Oven-dried shoots of both potatoes (Exp. 1) and carrots (Exp. 3b) were milled using a Retsch ZM 200 centrifuge mill (Retsch GmbH, Germany) and 0.2 g of each milled sample was digested in 6 mL of concentrated nitric acid via microwave digestion (Kumssa et al., 2017). Oven-dried carrot tap roots (Exp.3b) were digested using a different method. Tap roots were ground using a pestle and mortar and 0.2 g of each sample was digested overnight in 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide. Following this, samples were heated to 95^{o}C for 2 hours in heating blocks. Digested samples from both digestion methods were then diluted down to 20 mL and 50 mL, respectively, in MilliQ water. Samples were diluted again 10-fold before Induced Coupled Plasma Mass Spectrometry (ICP-MS) was carried out to determine the nutrient concentration of 31 elements, including macro- and micronutrients, in the plant material. The nutrient concentration of the plant material was then used to calculate the nutrient content of the plant shoots and roots. Shoot or tap root dry weight (kg) was multiplied by the nutrient concentration of the respective plant material (mg/kg) to find the nutrient content of the shoots and tap roots (mg).

2.4 *Bacillus amyloliquefaciens* QST 713 root colonisation assessment

2.4.1 Root sampling

At harvest, small sections of plant root with rhizospheric soil attached were sampled at various points down the soil profile, depending on the experiment. For each sample approximately 250-350 mg of soil, including approximately 2 cm of root, was collected into a 2 mL micro tube ready for DNA extraction. Sample weight was recorded before samples were frozen at -20^{o}C.

2.4.2 DNA extraction

DNA from the rhizospheric samples was extracted using the FastDNA^{TM} SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany). The protocol specified by the kit was followed exactly and each DNA sample was collected in 75 µL of DNase-free water. DNA samples were frozen at -20^{o}C.

2.4.3 Standard curve preparation

A standard curve for *B. amyloliquefaciens* was prepared whereby a series of tenfold dilutions of concentrated Serenade ASO® was made up from x10^{-1} to x10^{-9}. Soil was ground down using a pestle and mortar and 250 mg was added to nine Lysing Matrix E 2.0 mL tubes supplied in the soil extraction kit. One hundred microlitres of each dilution was added to each 2 mL tube of soil at the same time as 100 µL of each dilution was plated onto four LB agar plates. Inoculated LB agar plates were incubated at 28^{o}C for 24 hours before CFU were counted. DNA was extracted from the tubes containing the soil and Serenade ASO® dilutions using the FastDNA^{TM} SPIN Kit for Soil. Following extraction into 75 µL of DNase-free water, DNA samples were

frozen ready for qPCR. This process was repeated twice and averaged to give an accurate *B. amyloliquefaciens* standard curve. CFU counts were averaged over both standard curves and multiplied by 10 to find the CFU mL^{-1} of the Serenade ASO® dilution. CFU mL^{-1} values were logarithmically transformed for use in the standard curve. qPCR was carried out on the DNA samples and cycle threshold values (Cq) were plotted against Log CFU mL^{-1} to produce a standard curve for *B. amyloliquefaciens* (Figure 2-1). The amplification efficiency of the qPCR assay was determined using the equation $E=10^{(-1/slope)}$ (Ramakers et al., 2003). Maximum efficiency (100%) is where the qPCR product doubles perfectly each cycle, therefore the maximum value for E is 2. For this qPCR assay E= 1.974, so 97% efficiency is observed.

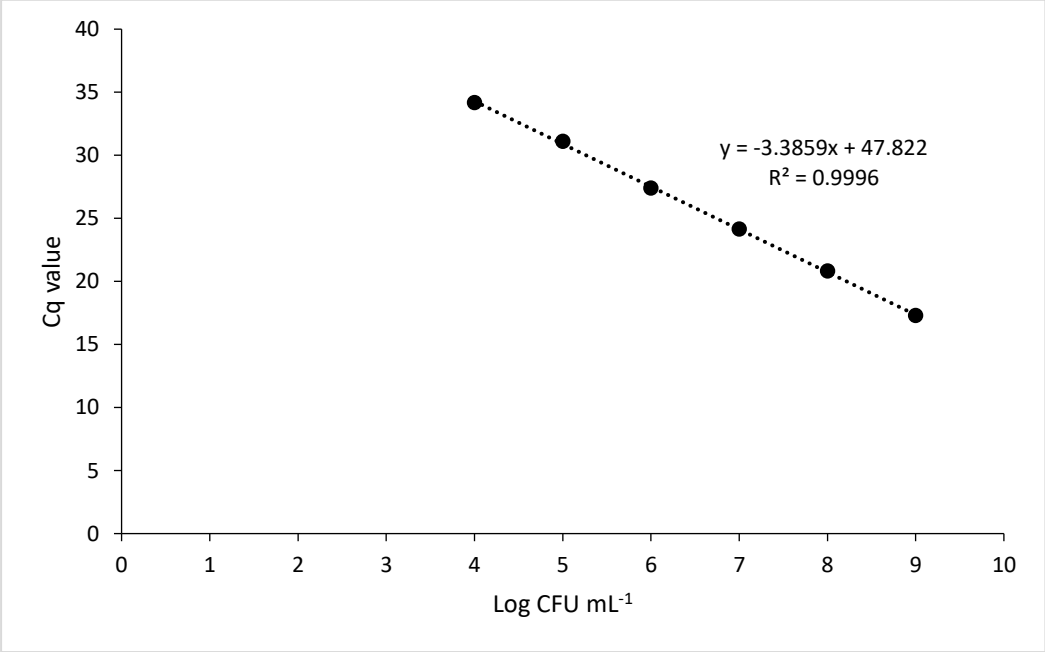

Figure 2-1: Standard curve for Bacillus amyloliquefaciens *QST 713.*

The standard curve equation and the Cq values of the rhizospheric samples taken from the various experiments were used to quantify *B. amyloliquefaciens* Log CFU mL^{-1} relatively in the rhizosphere of both potatoes and carrots. Log CFU mL^{-1} values for each sample were back transformed into CFU mL^{-1}. CFU mL^{-1} values were then divided by the weight of the soil sample in which they were extracted from to give the CFU g^{-1} of rhizospheric soil at the respective root depths.

2.4.4 qPCR

All DNA samples underwent qPCR using the Roche LightCycler480. PerfeCTa SYBR® Green FastMix by Quantabio was used alongside the *B. subtilis*-specific primers: forward (5'-GCG GCG TGC CTA ATA CAT GC-3') and reverse (5'-CTC AGG TCG GCT ACG CAT CG-3') (Lahlali et al., 2013). Each 20 µL qPCR reaction was made up of 2 µL DNA, 6.8 µL distilled water, 10 µL PerfeCTa SYBR® Green FastMix and 0.6 µL each of forward and reverse primers (standard concentration 10 µM). To check for inhibition by soil inhibitors, qPCR was carried out on both undiluted and diluted DNA samples from the standard curve. If uninhibited, the difference in Cq between 10-fold dilutions should be 3.3 (or $\log_2(10)$). Results revealed that PCR with undiluted DNA was inhibited, but PCR with DNA diluted tenfold was not. Therefore, all DNA samples, including standard curve samples, were diluted 10x before running the qPCR assay. Non-template controls used nuclease free water instead of DNA. A positive control was carried out using Universal 16S rRNA primers (Forward: 5'-GCG GCG TGC CTA ATA CAT GC-3', Reverse: 5'-CTC AGG TCG GCT ACG CAT CG-3') to confirm that amplification of 16S bacterial DNA was not inhibited resulting in false negative results. A standard curve was run on every qPCR plate as a positive control.

qPCR reactions were either duplicated or triplicated for each sample. The qPCR thermocycling conditions were as follows: 2 min at 95^{o}C, followed by 30 s at 95^{o}C, 30 s at 56^{o}C and 30 s at 72^{o}C for 45 cycles, followed by cooling at 40^{o}C. A melting (dissociation) curve was constructed after the last amplification cycle to ascertain the qPCR specificity. This was analysed using the Tm calling analysis within the Roche LightCycler® 480 software v 1.5. The melting temperature (Tm) for *B. amyloliquefaciens* was 78^{o}C+/- 1 and a single peak indicated that only *B. amyloliquefaciens* DNA was amplified. Cq values were analysed by Absolute Quantitative/2nd derivative Max analysis in Roche LightCycler® 480 software v 1.5. The criteria used to determine positive results were Cq values < 35 and positive Tm peaks at 78+/-1 ^{o}C. Results outside of this range were discounted and it was concluded *B. amyloliquefaciens* was not detected (ND). This assay only allowed accurate *B. amyloliquefaciens* detection down to 10^4 CFU mL^{-1} (Cq<35). Lower than this, results cannot confidently demonstrate that *Bacillus* is not present, but concentrations are too low for reliable detection, therefore ND.

Chapter 3 Experiment 1: The effect of *Bacillus amyloliquefaciens* QST 713 root colonisation on potato growth

3.1 Sub-hypothesis

- Root colonisation by *B. amyloliquefaciens* QST 713 stimulates early potato growth.

3.2 Materials and Methods

3.2.1 Experimental set up

This experiment consisted of three treatments and six replicates (reps), totalling 18 columns. Treatments included an untreated control (UTC) and two different methods of *B. amyloliquefaciens* QST 713 application. Seed potatoes (cv. Maris Piper) of a similar size were chosen and all shoots removed except one. Columns were prepared for sowing as described in section 2.1.1 and placed in 9 cm deep containers.

Bacillus amyloliquefaciens QST 713 treatment was prepared as described in 2.1.2. Five litres ha^{-1} of Serenade ASO® in 100 L of water equates to 1 µL of Serenade ASO® suspension per 1 cm^2 of soil surface and so using the soil surface area of 162.9 cm^2, the dose of Serenade ASO® suspension per column was 162.9 µL. Approximately 7.9 $x10^7$ CFU were applied per treated column.

UTC tubers were placed eye up onto the soil surface and covered in 8 cm of dampened soil to avoid having to water the top soil after sowing. To dampen the soil, the volume of soil required to fill the rest of the column was calculated to be 1302.88 cm^3, using the radius 7.2 cm and a depth of 8 cm. This was multiplied by a

bulk density of 1.2 g cm^{-3} to find a mass of 1.56 kg dry soil per column. Soil was weighed out and mixed with 100 mL of water before covering the tuber.

For the pipette treatment, a graduated pipette was used to apply 162.9 µL of Serenade ASO® suspension onto the soil surface directly beneath the tuber. The tuber was then placed, eye up, on top of the treated soil and covered with another 8 cm of dampened soil (as described for the UTC).

For the mixed treatment (2/3 mixed into soil, 1/3 pipette), the dose of Serenade ASO® suspension remained the same but it was not concentrated as close to the tuber. A graduated pipette was used to apply 54.3 µL of the dose (1/3 of the dose) directly underneath the tuber while the other 2/3 of the dose, 108.6 µL, was mixed into the 5 cm of soil above the tuber. To mix the Serenade ASO® suspension with the topsoil, all six replicates were prepared at once. The volume of treated soil required per column was 814.5 cm^3. This volume was multiplied by a bulk density of 1.2 g cm^{-3} to find the weight of dry soil required, calculated as 0.98 kg per column. All six columns worth of treated soil equated to 5.86 kg. 651.4 µL of Serenade ASO® suspension (6 doses), was added to 376 ml of water before being mixed evenly into the 5.86 kg of soil. Serenade ASO® suspension was added to water to allow for an even mix throughout the soil and to dampen the soil. The volume of water used was calculated at the same rate as the dampened soil in the UTC columns. The soil and Serenade ASO® suspension were mixed thoroughly and tubers were placed on the treated soil surfaces, eyes up. Treated soil was evenly distributed between the six columns before 3 cm of dampened soil was added on top of each column.

Columns were arranged in a randomised block design in a controlled environment growth room with a day time temperature of 20^{o}C and a night time temperature of 15^{o}C under a 16-hour photoperiod. The growth room was equipped with halogen bulb lighting at an intensity of approximately 11.7 µmol.m^{-2} s^{-1} at the soil surface. Potatoes were grown for 28 days and kept watered from the bottom to ensure that *B. amyloliquefaciens* was not leached down the column via water infiltration.

3.2.2 Growth parameter assessment and DNA analysis

3.2.2.1 Harvest processing

After 28 days, potato growth parameters were measured as described in 2.2.1. Rhizospheric samples were taken for DNA extraction from across the columns' width at four points down the column: at the top of column surrounding the tuber, 15 cm down the column, 30 cm down and at the bottom root tip. The soil columns were cut into volumes of 0-15 cm, 15-30 cm and 30-60 cm depth (2.2.1) ready for root washing.

3.2.2.2 Root assessment

At first, roots were hand washed and this was the case for Blocks 1, 2 and 5 for 0-15 cm and 15-30 cm. The rest of the soil samples (Blocks 3, 4 and 6, and 30-60 cm of blocks 1, 2 and 5) were washed using the Delta-T root washer (2.2.2). At this point the remaining tuber was discarded. Clean roots were scanned using WinRHIZO® scanning equipment and roots were scanned in smaller sections to allow roots to be spread out on the scanning plate. As described in 2.2.2, scanned roots were quantified and below ground dry weight was calculated.

3.2.2.3 DNA Extraction and qPCR

DNA extraction was performed on the rhizospheric soil samples collected at harvest (2.4.2) and qPCR was undertaken to quantify the *B. amyloliquefaciens* DNA present (2.4.4). qPCR reactions of samples in blocks 1-3 were duplicated and in blocks 4-6 were triplicated on the qPCR plate.

3.2.3 Statistical analysis

Growth parameter data was analysed in GenStat 17.0 via general ANOVA. During root washing, control samples of roots from blocks 1 and 2 were accidentally mixed together and so accurate data could not be collected. These two data points were therefore discarded and left as missing values which were accounted for by GenStat.

3.3 Results

Table 3-1: Average growth parameters of 28 day old potatoes treated with Bacillus amyloliquefaciens *QST 713.*

Treatment	Shoot height (cm)	Rooting depth (cm)	Shoot dry weight (g)	Below ground dry weight (g)	Total root length density (cm cm^{-3})	Average root diameter (mm)
UTC	30.8	60.7	3.35	0.608	0.979	0.305
2/3 mixed, 1/3 pipette	32.8	54.3	4.08	0.857	1.282	0.320
Pipette	36.0	56.5	4.84	0.968	1.526	0.319
***P*-value**	0.047	0.498	0.164	0.102	0.085	0.871
Fisher LSD (5%)	3.997	-	-	-	-	-

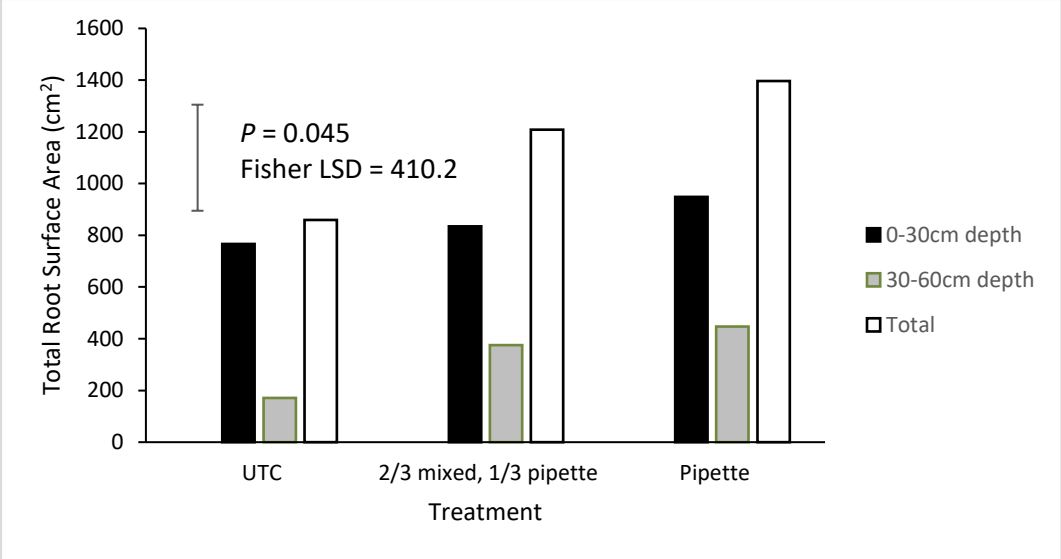

Figure 3-1: The effect of Bacillus amyloliquefaciens *QST 713 treatment on the surface area of 28-day old potato roots. The P-value and 5% Fisher LSD values are shown for the total root surface area of the whole plant.*

There was a general increase in potato growth in response to the volume of *B. amyloliquefaciens* QST 713 concentrated around the tuber (Table 3-1). Potatoes treated with *B. amyloliquefaciens* via pipette application had significantly taller shoots than untreated potatoes (*P*= 0.047). Most interestingly, *B. amyloliquefaciens* QST 713 treatment resulted in a significant increase in total potato root surface area when applied directly below the tuber via the pipette treatment (Figure 3-1). As the amount of *B. amyloliquefaciens* directly surrounding the tuber increased, the increase in root surface area was larger. This indicates that, although not a dose response, as the dose was the same in both treatments, there was a trend for a treatment concentration response. The closer *B. amyloliquefaciens* was situated to the tuber, the larger the root system and shoot height of the potato plant. The differences observed when the root system was split into 0-30 cm and 30-60 cm sections were not significant, although the graph suggests the biggest differences in root surface area were seen at the depth 30-60 cm. The increase in root surface area

observed suggests there is potential for increased nutrient uptake. *Bacillus amyloliquefaciens* appeared to have no effect on the average root diameter of potato roots or the average rooting depth.

Table 3-2: The macro- and micro-nutrient content[1] of 28-day old potato shoots treated with Bacillus amyloliquefaciens *QST 713.*

	B (mg)	Na (mg)	Mg (mg)	P (mg)	S (mg)	K (mg)	Ca (mg)	Ti (mg)	Li (mg)	Be (mg)	Al (mg)
UTC	0.120	0.066	11.3	13.1	18.0	179	63.1	0.0137	0.0009	3.7E-05	0.12
Mixed	0.156	0.099	13.8	15.8	21.2	228	75.00	0.0165	0.0012	1.0E-06	0.17
Pipette	0.172	0.153	15.7	18.00	24.1	269	90.8	0.0289	0.0040	0.0002	1.52
***P*-value**	0.188	0.006	0.287	0.294	0.288	0.08	0.257	0.02	0.03	0.027	0.052
Fisher LSD (5%)	0.059	0.046	5.85	6.58	8.04	78.8	35.05	0.0104	0.0024	0.0001	1.242

	V (mg)	Cr (mg)	Mn (mg)	Fe (mg)	Co (mg)	Ni (mg)	Cu (mg)	Zn (mg)	As (mg)	Se (mg)
UTC	0.0005	0.00084	0.122	0.64	0.0008	0.0018	0.0503	0.165	0.0002	0.0001
Mixed	0.0007	0.00113	0.144	0.80	0.0011	0.0025	0.0677	0.208	0.0003	0.0002
Pipette	0.0069	0.00627	0.232	4.74	0.0023	0.0061	0.0795	0.260	0.0025	0.0002
***P*-value**	0.057	0.026	0.028	0.063	0.032	0.023	0.132	0.128	0.065	0.023
Fisher LSD (5%)	0.0058	0.0041	0.0808	3.81	0.0011	0.0031	0.0293	0.0944	0.0021	8.67E-05

	Rb (mg)	Sr (mg)	Mo (mg)	Ag (mg)	Cd (mg)	Cs (mg)	Ba (mg)	Tl (mg)	Pb (mg)	U (mg)
UTC	0.083	0.165	0.0030	5.99E-05	0.0016	0.0002	0.0382	3.32E-05	0.0004	0.000015
Mixed	0.122	0.197	0.0037	7.66E-05	0.0022	0.0004	0.0473	4.91E-05	0.0007	0.000083
Pipette	0.147	0.248	0.0046	1.06E-04	0.0023	0.0006	0.0777	8.67E-05	0.0042	0.0002
***P*-value**	0.108	0.17	0.195	0.112	0.34	0.03	0.035	0.002	0.043	0.073
Fisher LSD (5%)	0.061	0.090	0.002	4.45E-05	0.0010	0.0003	0.0299	2.53E-05	0.0032	0.0002

[1] Nutrient content is a product of nutrient concentration (mg/kg) and shoot dry weight (kg)

Despite the increased root surface area of the potato roots, ICP-MS analysis revealed no significant differences in the macronutrient concentration (P, K, S, Mg and Ca) of the potato shoots (results not shown). However, significant increases in the Ti, Se, Cs and Tl concentration of pipette treated potato shoots were observed compared to untreated shoots. When the nutrient content of the leaves was calculated, which accounts for the nutrient dilution effect that occurs with increasing shoot biomass, a clear trend was observed in the nutrient content of the potato shoots (Table 3-2). As *B. amyloliquefaciens* QST 713 was applied closer to the tuber, the nutrient content and therefore the nutrient uptake of the potato shoots was increased. A significant increase in the elemental content of pipette treated shoots was observed in Na, Ti, Li, Be, Cr, Mn, Co, Ni, Se, Cs, Ba, Tl and Pb. Differences for other elements, including P, K, S, Mg, Ca, B, Al, V, Fe, Cu, Zn, As, Rb, Sr, Mo, Ag, Cd and U, although not significant, also showed a similar pattern for increased uptake following pipette treatment with *B. amyloliquefaciens* QST 713.

Table 3-3: Average Bacillus amyloliquefaciens *CFU g^{-1} detected in the potato rhizosphere at various depths[2].The number of positive reactions is out of a total of two reactions for replicates 1-3 and a total of three reactions for replicates 4-6.*

Treatment	Depth (cm)	Rep	Average CFU g^{-1} soil	No. positive reactions
Control	2	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Mixed	2	1	6.88E+04	2
		2	9.64E+04	1
		3	1.66E+05	1
		4	7.79E+04	2
		5	4.56E+04	3
		6	ND	0
Mixed	15	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Mixed	30	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Mixed	Root tip	1	ND	0
		2	ND	0
		3	3.47E+04	1
		4	ND	0
		5	2.41E+04	1
		6	ND	0

[2] *Bacillus subtilis* CFU g^{-1} values presented here are an average of the CFU values for each rep (not including ND results). ND results were determined by a Cq value >35 and/or a Tm value outside the range of 78 +/- 1 ^{o}C.

Treatment	Depth (cm)	Rep	Average CFU g^{-1} soil	No. positive reactions
Pipette	2	1	ND	0
		2	1.19E+05	1
		3	ND	0
		4	3.32E+05	3
		5	ND	0
		6	ND	0
Pipette	15	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Pipette	30	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Pipette	Root tip	1	1.36E+05	1
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0

Results showing the ability of *B. amyloliquefaciens* to colonise the potato root system were not consistent (Table 3-3) (raw qPCR data, Table 8-1: The quantity of *Bacillus amyloliquefaciens* (CFU g^{-1} of soil) found in the potato rhizosphere at various depths.). Where *B. amyloliquefaciens* was applied as the mixed treatment CFU counts were commonly 10^4 CFU g^{-1} at the top of the column, surrounding the tuber.

Moving further down the soil profile at 15 cm and 30 cm no *B. amyloliquefaciens* was detected in this assay. At the root tip *B. amyloliquefaciens* was again detected at 10^4 CFU g^{-1} in two replicates (one reaction each). Two samples taken for the pipette treatment at the top of the column, surrounding the tuber, had high CFU counts of approximately 10^5 CFU g^{-1} in at least one reaction, but fewer positive detections were observed compared to the mixed treatment. Again at 15 cm and 30 cm *B. amyloliquefaciens* was not detected and at the root tip one rep showed a positive detection at 10^5 CFU g^{-1} in one reaction. Multiple samples had *B. amyloliquefaciens* on the root system at levels of 10^3 CFU g^{-1}, but as Cq values were >35, results were deemed unreliable. All control samples were free of *B. amyloliquefaciens* DNA.

These results show that colonisation of the potato root system by *B. amyloliquefaciens* does occur, but in this assay detection was irregular. The most common place to find *B. amyloliquefaciens* was in the soil surrounding the tuber and at the bottom tip of the root system. Samples where *B. amyloliquefaciens* was surprisingly undetected were run with Universal 16S rRNA bacterial primers to check for inhibition and therefore false negative results. Results were positive, indicating that *B. amyloliquefaciens* was simply not detected via this assay.

3.4 Discussion

Multiple strains of *Bacillus* species have been investigated for their plant growth promoting effects, with growth benefits found in a number of crops (Yao et al., 2006), including potatoes (Meng et al., 2016). This experiment suggests that root colonisation by the *B. amyloliquefaciens* strain QST 713 results in stimulation of potato growth, 28 days into development. Treatment of potatoes with *B.*

amyloliquefaciens led to a more extensive root system with a significantly increased root surface area and although the differences were not significant, an increased root length density (*P*=0.085) and root dry weight (*P*=0.102). A similar increase in root system was also observed in the field after tubers were treated with *Bacillus* strain FBZ24®, a similar strain to QST 713. Kilian et al. (2000) observed a 6% increase in root fresh weight at the beginning of tuber formation as well as a subsequent 8% increase in yield following treatment. Meng et al. (2013) found that inoculation with another similar strain, *B. amyloliquefaciens* BAC03, caused an increase in tuber weight of up to 33% in the field, albeit under disease pressure as the potatoes were inoculated with *Streptomyces scabies* alongside BAC03. In an experiment focusing on growth promotion, Meng at al. (2016) found BAC03 to increase potato shoot height, shoot dry weight and root/tuber dry weight. The increased root system that we observed in treated potato plants at 28 days after sowing could therefore have the potential to lead to increased tuber yield. In another experiment investigating the effect of a strain of *B. amyloliquefaciens* on cotton seedlings, Irizarry and White (2017) found the bacteria to have a beneficial effect on root architecture. More specifically they significantly increased primary root length, lateral root number, lateral root length and shoot to root ratio, as well as producing thinner roots with increased branching. Similar to our experiment, inoculation led to increased root surface area and significantly increased cotton shoot height.

In our experiment, significantly taller shoots were observed in the treated potatoes and although not significant, a trend was observed for increased shoot dry weight (*P*=0.164) as *B. amyloliquefaciens* treatment was more concentrated around the

tuber. It therefore could be hypothesized the larger root system could cause increased nutrient uptake. Initial results from ICP-MS analysis gave the elemental concentration of the potato shoots and no significant differences were observed in macronutrient concentrations between treated and untreated shoots. However, when the differences in shoot dry weight were accounted for, a trend was observed for increased macronutrient content of treated potato shoots and a significant increase in the elemental content of pipette treated shoots could be observed for a number of elements, including micronutrients such as Mn, Ni and Se. The trend observed for increased nutrient uptake reflected the increased root system and change in root architecture observed in potato following *B. amyloliquefaciens* treatment.

This trend is in line with a study by Yao et al. (2006) who reported the ability of *Bacillus* strain FZB24, a similar PGPR strain, to improve the capacity of cotton root systems to mobilise and take up nutrients, resulting in increased plant growth and yield. Our results are broadly in agreement with Altuntas (2018), who found *B. amyloliquefaciens* QST 713 to be an effective biofertiliser, significantly increasing broccoli root and shoot dry weight and significantly increasing the nutrient uptake of essential elements such as N, K, Mg, Ca, Mn and Zn, resulting in significant increases in broccoli head weight, vitamin C content and overall yield. Similar strains GB03 and IN937a have also been seen to promote growth of tomato and pepper plants, improving root architecture in particular (Kloepper et al., 2007). In this study, inoculation was seen to increase root parameters such as root surface area, total root length, root diameter and the number of root tips, in turn increasing the ability

of plants to take up nutrients. Kloepper et al. (2007) suggests how increased surface area may increase the area of the soil explored and therefore the opportunity for nutrient uptake. This could explain the similar trends we observed in increased root surface area of *B. amyloliquefaciens* treated potatoes and increased nutrient uptake. Root surface area is an important component contributing to nutrient uptake as increases in lateral root and root hair proliferation enhance root architecture, increasing the opportunity for nutrient uptake (López-Bucio et al., 2003, Marschener, 1998). Kloepper et al. (2007) suggest that root surface area can be enhanced by the increased formation of lateral roots and root hairs. As differences in root hairs cannot be detected via WinRHIZO scanning we could conclude that an increase in lateral root proliferation may therefore have caused the increase in root surface area seen in treated potatoes. However, further work to visualise the potato root system in detail is required to understand this.

As discussed in 1.3.3.2, *B. amyloliquefaciens* is known for its phosphate solubilising ability (Borriss, 2011). Phosphate solubilisation has therefore been thought to be partly responsible for the growth promotion effects exhibited by *Bacillus* species in multiple studies (Idriss et al., 2002, García-López et al., 2016, Garcia-Lopez et al., 2018, Singh and Satyanarayana, 2011). Studies have found treatment with *B. amyloliquefaciens* QST 713 to increase phosphate uptake in cucumber plants by 40%, significantly increasing P content of roots and shoots (García-López et al., 2016), as well as in wheat (Garcia-Lopez et al., 2018). Changes in the phosphate content of *B. amyloliquefaciens* QST 713 treated plant shoots were therefore expected in this experiment; however, significant increases in the phosphate content of potato

shoots were not observed, only a trend for an increased P content in treated plants, similar to that seen in S, K, Ca and Mg. This may suggest that the enhanced growth may not have been directly due to phosphate solubilisation, although it may have been a contributing factor. As discussed previously, other potential causes of growth promotion might include phytohormone production (Cabra Cendales et al., 2017, Idris et al., 2007), or production of other secondary metabolites such as VOCs (Borriss, 2011). Looking into the specific modes of action causing enhanced potato growth was beyond the scope of this study. However, the pattern we observed in the increased potato root system mirrors the pattern seen in shoot nutrient uptake, suggesting a causal effect. Further work is required, growing potatoes through to full development to investigate the effects of *B. amyloliquefaciens* QST 713 on tuber growth and nutrient content.

The trend in potato growth and nutrient content results suggest that the closer *B. amyloliquefaciens* QST 713 was applied to the tuber, the larger the observed growth promotion effects were. This suggests that treating tubers directly with *B. amyloliquefaciens* QST 713 could be beneficial. Ozer and Bayraktar (2014) applied *B. amyloliquefaciens* QST 713 as a seed tuber treatment to protect against soil-borne *Rhizoctonia* species and reported similar control efficacies to chemical fungicides, suggesting its potential as an effective seed treatment. However, Kilian et al. (2000) found that applying the similar strain *Bacillus* FBZ24 as a foliar spray increased the growth promotion effect slightly from that of seed treatment, increasing tuber yields. Further experimental work into applying *B. amyloliquefaciens* QST 713 as

both a seed treatment and a foliar spray for growth promotion could therefore be another avenue of research.

It is evident from qPCR results that colonisation of potato roots by *B. amyloliquefaciens* QST 713 was successful, but the extent of root colonisation and the pattern in which colonisation occurred was hard to assess. Due to the small sample number and size associated with collecting rhizosphere samples for DNA extraction the amount of the root system assessed for the presence of *B. amyloliquefaciens* DNA was limited. The very large potato root system meant only a small proportion could be sampled and so *B. amyloliquefaciens* QST 713 may have been present at certain depths, just not where we sampled. It may also have been that concentrations of *B. amyloliquefaciens* were too low for detection via this assay. The most common depths to find *B. amyloliquefaciens* were at the top of the root system and on the root tips at the bottom of the root system. It is not surprising that *B. amyloliquefaciens* was found most commonly at the top of the column but detecting *B. amyloliquefaciens* at the root tip was not expected. As our experiment was designed to water potatoes from the bottom, no water infiltration occurred and so the downwards movement of *B. amyloliquefaciens* was not due to leaching of the bacteria through the soil. Instead it could either be put down to the downwards growth of the colonised root apex (Liddell and Parke, 1989), or the movement of *B. amyloliquefaciens* down the root surface by the formation of biofilms, via swimming or swarming, or via flagella-independent migration (Bowen and Rovira, 1999, van Gestel et al., 2015). Although water infiltration was excluded in this experiment, it is considered an important mechanism for the colonisation of plant roots by PGPR

(Liddell and Parke, 1989). The pattern of colonisation observed in this experiment has also been observed in other PGPR strains. *Bacillus amyloliquefaciens* FBZ42 has been observed to preferentially colonise the root tips (Fan et al., 2012) and the lateral roots (Dietel et al., 2013) of Arabidopsis. *Bacillus* strain FBZ24 was also found to populate the root tips of peas more densely than the middle or top of the roots. This is thought to be due to root tips being very biologically active (Kilian et al., 2000). Fan et al. (2012) found that maize root tips were not favourable to FBZ42 colonisation and suggested this was due to the rapid growth rate of the roots exceeding the spreading ability of the bacteria. This may explain why *B. amyloliquefaciens* was not detected in the middle layers of the potato root system. This may have also been due to sampling of the primary roots rather than the lateral roots as the study by Dietel et al. (2013) suggests colonisation may be more common on lateral roots than primary roots.

Other bacterial extraction methods use the whole root system or a larger proportion of the root system to assess root colonisation. For example, studies by Ben Abdullah et al. (2018) and Chowdhury et al. (2013) used dilution plating and CFU counts to determine the effectiveness of colonisation across a larger surface area of tomato and lettuce (5 g of lateral roots and rhizosphere soil) root systems respectively. The study by Fan et al. (2012) discussed previously used a *Bacillus* strain labelled with a green fluorescent protein alongside confocal laser scanning microscopy and electron microscopy to target small areas of root, similar to those in our experiment, or the whole root system of very small seedlings (maize). This method shows visually the pattern and breadth of root colonisation as it can be visualised via fluorescence.

However, it is also limiting due to the small area of the root system that is assessed. Microscopy has the benefit of visualising root hairs, which might have been useful in this study to understand if root hair proliferation was a contributor to the increased root surface area. However, it does not give a quantitative measure of colonisation, which is an advantage of qPCR and CFU plating.

Overall, results from this experiment showed that *B. amyloliquefaciens* QST 713 treatment had a positive effect on potato growth after 28 days. Increases in root growth and above ground biomass were observed following QST 713 treatment, as well as a trend for increased nutrient uptake. These results combined with previous studies indicate the potential for yield benefits following *B. amyloliquefaciens* QST 713 treatment. Further work must go into testing various application methods for *B. amyloliquefaciens* QST 713, particularly for its use as either a seed treatment or a foliar spray, with a view to finding the optimal field application method. Another avenue of research which still requires detailed investigation is into the specific modes of action which cause QST 713 to change plant morphology, particularly root architecture.

Chapter 4 Experiment 2: The effect of *Bacillus amyloliquefaciens* QST 713 on early carrot growth

This chapter describes two experiments looking at the effects of *B. amyloliquefaciens* QST 713 root colonisation at different stages of early carrot growth. Results from Experiment 1 found that the closer *B. amyloliquefaciens* was applied to the tuber the more potato growth was stimulated. Following on from this, Experiment 2b investigated various application methods, allowing assessment of any differences observed in the root colonisation and growth promotion effects exhibited by *B. amyloliquefaciens* QST 713 via these different methods.

4.1 Sub-hypotheses

- Root colonisation by *B. amyloliquefaciens* QST 713 stimulates early carrot growth.
- The method used to apply *B. amyloliquefaciens* QST 713 affects carrot root colonisation and bio-stimulation.

4.2 Materials and Methods

4.2.1 Experiment 2a: 21-day old carrot seedlings

4.2.1.1 Experimental set-up

Small columns were prepared for sowing as described in 2.1.1. This experiment involved two treatments: six untreated carrots and six carrots treated with *B. amyloliquefaciens* QST 713. Carrot seeds (cv. Nairobi F1) were placed on the prepared seed bed of each column. Untreated seeds were covered with 5 mm of damp soil. Serenade ASO® suspension was made up as in 2.1.2 and the dose per column was calculated using the soil surface area, 44.2 cm^2. Ten litres ha^{-1} of Serenade ASO® in 200 L of water equates to 2 µL of Serenade ASO® suspension per 1

cm^2 of soil surface and so 88.8 µL of Serenade ASO® suspension was pipetted directly beneath the seeds in each treated column, before being covered with 5 mm of damp soil. The total *B. amyloliquefaciens* CFU added per column was approximately 4.35 $x10^7$ CFU. If multiple seedlings emerged they were removed to leave one seedling per column and columns were watered from the bottom. The experiment was set up in a randomised block design in a controlled environment growth room where the temperature was 20°C during the day and 15°C during the night with a 16-hour photoperiod. The growth room was equipped with halogen bulb lighting at an intensity of approximately 6.6 µmol.m^{-2} s^{-1} at the soil surface.

4.2.1.2 Growth assessment and root colonisation quantification

After 21 days, carrot seedlings were measured and quantified as described in 2.2.1. Rhizospheric samples, including approximately 1 cm of root, were taken from the tap root at the top (2 cm), the middle (13 cm) and the bottom (25 cm), as described in 2.4.1. Roots were hand washed and quantified using the WinRHIZO scanner (2.2.2). DNA extraction and qPCR were performed on the rhizospheric samples to determine *B. amyloliquefaciens* colonisation of the carrot root system (2.4).

4.2.1.3 Statistical Analysis

Seedling growth parameters were analysed using a general ANOVA in GenStat 17.0.

4.2.2 Experiment 2b: 37-day old carrots

4.2.2.1 Experimental set-up

To test the application method most suitable for *B. amyloliquefaciens*, this experiment comprised five different treatments, including an untreated control. Four different application methods were used to apply the same dose of *B.*

amyloliquefaciens QST 713 (Serenade ASO®). Carrots (cv. Nairobi F1) were grown in larger columns, which were prepared for sowing as described in 2.1.1. Seeds were sown four per column and were either left untreated or treated with Serenade ASO®. Serenade ASO® suspension was made up as in 2.1.2 and the dose per column was calculated using the soil surface area of 162.9 cm^2. Ten litres ha^{-1} of Serenade ASO® in 200 L of water equates to 2 µL of diluted Serenade ASO® suspension per 1 cm^2 of soil surface and so the dose per column was 325.8 µL. This was approximately 1.6 $x10^8$ *B. amyloliquefaciens* CFU per column.

The pipette treatment used a graduated pipette to apply 325.8 µL of Serenade ASO® suspension directly onto the seedbed surrounding the carrot seeds before being covered with 5 mm of soil. In the mixed treatment, 325.8 µL per column of Serenade ASO® suspension was mixed evenly into 5 mm of topsoil covering the seeds. For this treatment the volume of soil required to cover all six columns was calculated and multiplied by a bulk density of 1.2 g cm^{-3}, to find a weight of 81.43 g of soil. The dose per column was multiplied by six which amounted to 1954.3 µL of Serenade ASO® suspension. This volume was mixed into 3 mL of water and then mixed with the soil to allow an even spread of bacteria throughout the soil. From this treated soil, 1/6 was used to cover the carrot seeds in each column of the mixed treatment. For the spray treatment, the seedbed was sprayed with Serenade ASO® suspension before sowing and carrot seeds were then covered with 5 mm of soil. For the one true leaf (TL) spray treatment, untreated carrot seeds were sown and covered with 5 mm of soil. At 1TL seedlings were sprayed with Serenade ASO® suspension. Both treatments were applied using a Berthoud knapsack sprayer delivering Serenade ASO® at a rate

of 10 L ha^{-1} in 200 L of water using a Hypro Evenspray E80-02 yellow nozzle at the operating pressure of 3 bar. The knapsack was calibrated with Serenade ASO® suspension at a concentration of 50 mL Serenade ASO® L^{-1} of water using the equation below.

Application (L ha^{-1}) = (600 x sprayer output (ml min^{-1})) ÷ (swath width (m) x forward speed (km h^{-1}))

(600 x 0.75) ÷ (0.90 x 2.454) = 203.7489 L ha^{-1}

A forward speed of 2.454 km h^{-1} was achieved by walking to a metronome set at 58 bpm and the sprayer nozzle was held 50 cm above the columns to deliver 203.7L ha^{-1} Serenade ASO® suspension. To confirm the calibration, filter paper discs were weighed before and after spraying to assess the amount of Serenade ASO® suspension hitting the surface. No treatment was applied to carrot seeds in the untreated control.

Using a randomised block design, carrots were grown in a controlled environment growth room at a daytime temperature of 20°C and a night time temperature of 15°C in a 16-hour photoperiod. The growth room was equipped with halogen bulb lighting at an intensity of approximately 5.8 µmol.m^{-2} s^{-1} at the soil surface. Carrots were watered daily from above.

4.2.2.2 Growth measurements and rhizosphere sampling

After 37 days of growth, carrots were harvested and measured as described in 2.2.1. Rhizospheric samples were taken from the tap root at 2 cm, 10 cm, 20 cm and at the bottom tip, as described in 2.4, and DNA extraction and qPCR were performed to

determine *B. amyloliquefaciens* root colonisation at these depths. Soil layers were collected in 3 sections: 0-15 cm, 15-30 cm and 30-45 cm; the rest of the column was discarded. Roots were recovered from these sections using the root washer and quantified using WinRHIZO® software (2.2.2). Unfortunately, after root washing it became evident that roots had begun to break down and so root growth quantification was not possible.

4.2.2.3 Statistical Analysis

Carrot growth parameters were analysed using a general ANOVA in GenStat 17.0. WinRHIZO root data could not be analysed.

4.3 Results

Table 4-1: Average above and below ground growth parameters of 21-day old carrot seedlings treated with Bacillus amyloliquefaciens *QST 713.*

Treatment	Root Depth (cm)	Shoot Height (cm)	Shoot dry weight (g)	Root dry weight (g)	Total root length (cm)	Total root surface area (cm^2)	Average root diameter (mm)	Root length density ($cm\ cm^{-3}$)
UTC	20.6	3.78	0.0045	0.0026	140	11.09	0.2531	0.1271
Treated	22.0	3.25	0.0041	0.0023	108	8.30	0.2439	0.0973
P-value	0.779	0.033	0.155	0.498	0.171	0.125	0.503	0.171
Fisher LSD	7.96	0.4678	0.00057	0.00123	53	3.901	0.0327	0.04796

In experiment 2a, treatment with *B. amyloliquefaciens* QST 713 did not improve carrot seedling growth 21 das (Table 4-1). In fact, UTC seedlings were significantly taller than treated seedlings (*P*=0.033). No significant differences were observed across the other growth parameters measured and so it appears treatment with *B. amyloliquefaciens* QST 713 does not enhance carrot seedling growth at this early stage.

Table 4-2: Average Bacillus amyloliquefaciens *CFU g^{-1} detected in the rhizosphere of 21-day old carrot seedlings at various depths[3]. The number of positive detections is out of a total of three qPCR reactions per replicate.*

Treatment	Depth	Rep	Average CFU g^{-1}	No. positive detections
Control	2	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Treated	2	1	1.16E+07	3
		2	1.77E+06	3
		3	5.18E+06	3
		4	3.42E+06	3
		5	3.42E+06	3
		6	1.43E+06	3
Treated	13	1	ND	0
		2	4.95E+04	1
		3	ND	0
		4	ND	0
		5	1.85E+05	1
		6	ND	0
Treated	25	1	ND	0
		2	ND	0
		3	5.66E+05	1
		4	ND	0
		5	ND	0
		6	ND	0

Root colonisation results after 21 days suggest that *B. amyloliquefaciens* colonised the carrot rhizosphere successfully at a depth of 2 cm down the tap root (Table 4-2) (raw data, Table 8-2). *Bacillus amyloliquefaciens* was detected in the top section of every treated seedling at densities as high as 1.44×10^7 CFU g^{-1}. *Bacillus amyloliquefaciens* was detected at a depth of 13 cm in two seedlings at slightly

[3]*Bacillus subtilis* CFU g^{-1} values presented here are the average of the values for each rep (not including ND results). ND results were determined by a Cq value >35 and/or a Tm value outside the range of 78 +/- 1°C.

reduced densities and at the root tip of one seedling at 5.66 x10^5 CFU g^{-1}. These results show a trend for *B. amyloliquefaciens* being present in high quantities at the top of the root system, where it is applied, and in decreasing amounts further down the root system at 21 days after sowing. As the carrots were watered from the bottom, it is evident that *B. amyloliquefaciens* was able to colonise roots down the rhizosphere and that detection was not due to leaching of the bacteria down the column following watering.

Table 4-3: The average growth parameters of 37-day old carrots treated with Bacillus amyloliquefaciens *QST 713 via multiple application methods.*

Treatment	Shoot height (cm)	Shoot dry weight (g)	Leaf area (cm^2)	Root depth (cm)
UTC	10.7	0.0636	7.07	43.5
Pipette only	11.7	0.0711	8.56	50.1
Mixed with soil	11.3	0.0667	7.17	45.5
Spray seedbed	11.4	0.0693	9.09	44.0
Sprayed at 1TL	13.0	0.0767	9.01	49.5
P-value	0.693	0.959	0.836	0.129
Fisher LSD (5%)	3.413	0.0372	4.925	6.405

In experiment 2b, no significant differences between treatment methods were observed in the growth parameter data collected on the 37 day old carrots at harvest (Table 4-3). As previously discussed, no root data is available.

Table 4-4: Average Bacillus amyloliquefaciens *CFU g^{-1} detected in the rhizosphere of 37-day old carrots at various depths following different application methods[4]. The number of positive reactions is out of a total of three qPCR reactions per replicate.*

Treatment	Depth	Rep	Average CFU g^{-1}	No. positive reactions
Control	2	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Mixed	2	1	5.55E+04	2
		2	ND	0
		3	1.05E+05	3
		5	ND	0
		6	5.99E+04	2
Mixed	10	1	1.84E+04	1
		2	ND	0
		3	ND	0
		5	ND	0
		6	ND	0
Mixed	20	1	ND	0
		2	ND	0
		3	ND	0
		5	ND	0
		6	ND	0
Mixed	Bottom	1	ND	0
		2	ND	0
		3	ND	0
		5	1.77E+04	1
		6	2.96E+04	2
Pipette	2	1	7.63E+05	1
		2	3.28E+04	1
		3	4.72E+05	3
		5	ND	0
		6	1.92E+05	1
Pipette	10	1	ND	0
		2	ND	0
		3	ND	0
		5	ND	0
		6	ND	0

[4] *Bacillus subtilis* CFU g^{-1} values presented here are an average of the CFU values for each rep (not including ND results). ND results were determined by a Cq value >35 and/or a Tm value outside the range of 78 +/- 1°C.

Treatment	Depth	Rep	Average CFU g^{-1}	No. positive reactions
Pipette	20	1	ND	0
		2	ND	0
		3	ND	0
		5	ND	0
		6	ND	0
Pipette	Bottom	1	9.86E+04	3
		2	2.57E+04	2
		3	ND	0
		5	ND	0
		6	ND	0
Spray	2	1	1.10E+05	3
		2	ND	0
		3	ND	0
		4	ND	0
		5	3.35E+05	2
		6	4.83E+04	2
Spray	10	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
Spray	20	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	4.01E+04	2
		6	1.53E+04	1
Spray	Bottom	1	2.81E+04	1
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
1TL	2	1	9.82E+05	3
		2	ND	0
		3	8.27E+04	3
		4	ND	0
		5	3.67E+04	1
		6	2.36E+05	3

Treatment	Depth	Rep	Average CFU g^{-1}	No. positive reactions
1TL	10	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
1TL	20	1	ND	0
		2	ND	0
		3	ND	0
		4	ND	0
		5	ND	0
		6	ND	0
1TL	Bottom	1	3.08E+04	2
		2	3.49E+04	1
		3	ND	0
		4	ND	0
		5	2.13E+04	3
		6	ND	0

qPCR results (Table 4-4) (raw data, Table 8-3) suggest that even though *B. amyloliquefaciens* detection was not consistent, *B. amyloliquefaciens* QST 713 was able to colonise right down the carrot root system. The majority of positive detections were in the top 2 cm of the tap root and at the bottom tip of the tap root. In all treatments *B. amyloliquefaciens* CFU g^{-1} appeared to decrease by approximately an order of magnitude from the top 2 cm of the column (approx. 10^4 or 10^5 CFU g^{-1}) to the bottom tip (approx. 10^4 CFU g^{-1}). *Bacillus amyloliquefaciens* detection in the middle of the column at depths 10 cm and 20 cm was uncommon. Although it is difficult to assess which application method resulted in the most consistent colonisation, it appears that the highest level of colonisation occurred in the 1TL treatment, with one replicate in the top 2 cm harbouring an average of $9.82x10^5$ CFU g^{-1} (Table 4-4).

4.4 Discussion

These experiments were carried out to determine whether stimulation of carrot growth could be achieved by *B. amyloliquefaciens* QST 713, and if so, how the treatment should be applied to achieve optimum results. Preliminary work (unpublished) suggested a trend for *B. amyloliquefaciens* QST 713 treatment to increase the root system of carrot seedlings, increasing lateral root proliferation in the early stages of development while reducing the primary root length. This pattern was also observed by Asari et al. (2017), who found treatment with *B. amyloliquefaciens* to cause an increase in the total root system development of Arabidopsis seedlings after 11 days of growth by increasing lateral root number, lateral root elongation and root hair proliferation. This change in root architecture also resulted in a reduction in primary root length by up to almost 50% and a significant increase in shoot biomass. Asari et al. (2017) suggested that plant hormones such as auxin and cytokinin contributed to the changes in root architecture observed in their study, although production of these hormones was not thought to be fully responsible. The promising preliminary results, however, were not replicated in this experiment and growth promotion of carrot seedlings by *B. amyloliquefaciens* QST 713 was not observed at 21 days following the application of 4.35 x10^7 CFU directly onto the seed. Interestingly, Meng et al. (2016) found strain BAC03, a similar strain to QST 713, to inhibit carrot seed germination by more than 90% when applied at rates of 10^7 CFU cm^{-3} of potting mix. Seedling growth was also inhibited and inhibition increased with BAC03 dose rate, slowing carrot seedling growth by >70% at the application rate 10^7 CFU cm^{-3}. In our 21-day carrot seedling

experiment, although no germination inhibition was observed, a slight trend (not significant) for untreated seedlings to grow marginally larger than treated seedlings was observed. Although the inhibition observed by Meng et al. (2016) at the seedling stage was only tested in strain BAC03 and not QST 713, they are similar strains and so this could be a potential explanation for the results we observed. However, it cannot be conclusive and more detailed research into the potential inhibition of germination and seedling growth by strain QST 713 should be carried out.

Similarly to Experiment 2a, no growth promotion was observed in treated carrots grown for 37 days (Exp. 2b). The only slight trend observed was the increase in rooting depth when *B. amyloliquefaciens* was applied via the pipette treatment and the 1TL spray treatment. The effect of *B. amyloliquefaciens* treatment on carrot root architecture could not be assessed, however, which limited the conclusions that could be drawn. Multiple studies describe the beneficial effects of *Bacillus* species on root architecture specifically (Irizarry and White, 2017, Kloepper et al., 2007) and so although differences were not observed above ground, differences in root architecture might have become apparent. A major improvement to this experiment would have been to minimise the time taken between harvesting the carrots and processing the roots. Due to the number of samples, the protocol used to process the carrot roots and root processing not being prioritised, the roots began to break down before quantification could take place.

The lack of a growth promotion effect observed in carrots after 37 days was surprising as multiple studies describe the beneficial effects of QST 713 or similar *Bacillus* strains on crop growth, as discussed in Chapter 1. Meng et al. (2016) found

that after six weeks of radish growth, QST 713 applied at a rate of 10^5 CFU cm^{-3}, 10 days after sowing, resulted in significant increases in radish shoot and root fresh and dry weight. Application of BAC03 was also found to have a highly significant effect on carrot growth after 8 weeks, resulting in significantly increased plant height and root and shoot dry weight. In a recent study by Ben Abdullah et al. (2018) tomatoes were treated with *B. amyloliquefaciens* subsp. *plantarum* strain 32a, another strain similar to QST 713. Three days after planting, each tomato plant was drenched with 10 mL of vegetative cell or spore suspension of strain 32a at a rate of 10^8 CFU ml^{-1}. This resulted in significant increases in germination, root and shoot length and root and shoot fresh weight.

Factors which could have been responsible for the lack of growth promotion observed in our two carrot experiments could have been the rate at which QST 713 was applied, combined with the application timing and the application technique used (Borriss, 2016). Our experiments used the recommended UK field dose rates, as the aim was to bring the application methods used under controlled environment conditions up to field scale application. Serenade ASO® bacterial suspension containing 4.9 x10^8 CFU mL^{-1} was applied at a rate of 20 mL m^{-2} and the volume required was calculated using the surface area of the soil in the column. The study described above by Ben Abdullah et al. (2018) drenched each tomato plant with approximately 10^9 CFU, three days after planting, compared to our 21-day and 37-day experiments which added approximately 4.35 x10^7 CFU and 1.6 x10^8 CFU directly onto the un-germinated seed, respectively. This suggests that the lack of growth promotion may have been due to the application rate, the timing, or the way in

which the treatment was applied, directly onto the seed, rather than via a root drench. The study by Meng et al. (2016) used a slightly reduced concentration of *Bacillus* (10^7 CFU ml^{-1}), also added as a root drench, but the volume added resulted in a final *Bacillus* concentration of 10^5 CFU cm^{-3} of potting mix in a 1 L pot. Therefore, each pot contained approximately 10^8 CFU. This was a tenfold increase from that added to each column in our 21-day experiment (4.35 x10^7 CFU), but it was similar to the amount added in our 37-day experiment, 1.6 x10^8 CFU, and so application rate may not have been the limiting factor in these experiments. The application technique and timing may still have been a limitation, however.

In the field experiment by Meng et al. (2013) described in Chapter 1, *B. amyloliquefaciens* was added at a rate of 1 L m^{-2} at a concentration of 10^6 CFU mL^{-1}, a total of four times at 2 week intervals, resulting in a significant potato yield increase. This increase in application frequency may therefore be a contributing factor to the beneficial effects observed with BAC03. The rate of bacterial solution application was similar to our experiment, which was 20 mL m^{-2} at a concentration of 4.9 x10^8 CFU mL^{-1}, only the volume we applied was lower but harboured a higher concentration of bacterial CFU. Similarly, in an experiment by Altuntas (2018) on the growth promotion effect of *B. amyloliquefaciens* QST 713 on broccoli plants, treatment was split into three doses with growth parameters such as leaf number, shoot fresh weight, and root dry weight being affected most after the third *B. amyloliquefaciens* QST 713 application (70 days) rather than the second (40 days). Treatment here was applied at the rate of 1 L per application. These experiments demonstrate the promising benefits of applying *Bacillus* strains multiple times. In agreement, Meng et

al. (2016) also found that increasing the application rate of BAC03 and the application frequency to more than one application resulted in significantly increased growth promotion effects. This is also in line with a study by Kokalis-Burelle et al. (2006), who found that increasing the number of applications of *B. subtilis* GB03 and *B. amyloliquefaciens* IN9371 did not increase the density of *Bacillus* colonisation, but it did cause a significant increase in shoot height and shoot and root dry weight of *Capsicum annuum*. Although the results discussed here were found for differing *Bacillus* strains, they might indicate that increasing the application rate and the application frequency of QST 713 could result in increased chances of a growth promotion response in carrots.

It is hard to conclude why carrot growth promotion was not observed after both 21 days and 37 days in these experiments. To the best of our knowledge there are no studies assessing the direct growth promotion effects of *B. amyloliquefaciens* strain QST 713 on carrots specifically and so only comparisons with similar strains and other crops can be made. Nowak (1998) describes how inoculation of beneficial bacteria is dependent on not only the plant species, but the variety and the growth conditions, as all these factors can affect physiological properties such as root exudate production and in turn, the plant-microbe interaction. Therefore, it may be that QST 713 does not interact with carrot roots in the same way that it appears to with other crops, such as radish, maize, cucumber and wheat (Accinelli et al., 2018, García-López et al., 2016, Garcia-Lopez et al., 2018, Meng et al., 2016). However, due to the lack of root development data at 37 days, a conclusion such as this cannot be made.

Detection of *B. amyloliquefaciens* QST 713 down the carrot tap root was successful in both experiments, indicating that *B. amyloliquefaciens* was able to colonise carrot roots after 21 days and 37 days of growth. On the 21-day old carrot roots, *B. amyloliquefaciens* was detected in higher densities at the top of the root system with detection rates and densities decreasing with depth. This pattern of colonisation is in line with the observation of van Elsas et al. (1986) who found *B. subtilis* populations to decrease with depth down the soil profile. This is also in agreement with Schmidt et al. (2004) who, in a similar column experiment, found the number of recovered *B. subtilis* MBI 600 spores to decrease with depth down the root system of sugar beet seedlings. At 1TL and a root depth of 2-4 cm, *B. subtilis* levels were at approximately 10^5 CFU g^{-1}, but at 4-6 cm, levels were as low as 10^1 CFU g^{-1}, which was near the detection limit of the assay. A study by Fan et al. (2012) also found that on week old maize seedling roots grown *in vitro*, the most abundant root colonisation by *B. amyloliquefaciens* FBZ42 was in the top 2-8 cm of the root system with bacterial density decreasing down the root until few were observed on the root tip. Colonisation appeared highest in the region where the majority of lateral roots emerged, *B. amyloliquefaciens* commonly being found at junctions between primary and lateral roots and on root hairs (Fan et al., 2012). The pattern observed in these experiments could be explained by the aerobic nature of *B. subtilis*, as it is thought to survive best in the top 1-3 cm of soil (Harwood and Cutting, 1990). *Bacillus subtilis* has been described as a strict aerobe (Borriss, 2015) and so the reduced detection of *B. amyloliquefaciens* at depth in our experiment (Exp. 2a) may have been due to slightly waterlogged and therefore anaerobic conditions at the bottom of the columns. This was due to standing the columns in saucers of water instead of

watering the carrots from above. Watering the columns from the bottom does, however suggest that *B. amyloliquefaciens* colonisation was successful right down the carrot seedling root system and was not due to washing of the bacteria down the column by water infiltration, as seen in the study by Liddell and Park (1989).

At 37 days a similar pattern of colonisation was observed on carrot roots to that of the potato roots (Exp. 1), in that the most common place for *B. amyloliquefaciens* detection was at the top of the carrot tap root and at the bottom root tip. As discussed in Chapter 3, this pattern of colonisation was also seen in the study by Fan et al. (2012), where *B. amyloliquefaciens* was observed via microscopy to colonise the root tips of Arabidopsis seedlings. No obvious differences were observed in the ability of *B. amyloliquefaciens* QST 713 to colonise carrot roots following the different application methods used in this experiment. Borriss (2016) suggests that application method is an important factor affecting efficacy and that microbial inoculants may have increased persistence when applied to younger plants as the native microbial population in the rhizosphere is not yet stable, meaning an improved chance of colonisation. This is challenged, however, by our results, which found similar colonisation success in carrots treated at 1TL to those treated at sowing.

The lack of root development data collected in Experiment 2b combined with the rhizosphere colonisation results, which did not suggest any obvious differences between the *B. amyloliquefaciens* QST 713 application methods, means that no strong conclusions can be drawn as to which application method provided the best results. Further work therefore must be carried out to determine conclusively

whether root colonisation by *B. amyloliquefaciens* QST 713 does in fact lead to improved root architecture, and if so, an effective application method must also be found in order to develop realistic field application methods.

Chapter 5 Experiment 3: The effect of temperature on *Bacillus amyloliquefaciens* QST 713 root colonisation and carrot growth

In the previous chapter a growth promotion effect by *B. amyloliquefaciens* QST 713 was not found in carrot growth at 21 days or in above ground growth or rooting depth at 35 days. Following on, this chapter investigates whether any growth promotion effects could be observed 70 days into carrot development. As no significant differences were detected between the application methods used in Experiment 2b, and the pipette treatment appeared to have a significant impact on potato growth in Experiment 1, a pipette was used to treat the carrots with QST 713 in the following experiment. The following experiment was also designed to look at the effect of lower temperatures on *B. amyloliquefaciens* root colonisation. Prior to this, an experiment was carried out to explore *B. amyloliquefaciens* growth at multiple temperature combinations on agar plates before investigating its interaction with plants.

5.1 Experiment 3a – Growth on agar plates

5.1.1 Sub-hypotheses:

- *Bacillus amyloliquefaciens* spores do not germinate at low temperatures when cultured on agar plates.
- Diurnal temperature changes affect *B. amyloliquefaciens* germination and growth rate.

5.1.2 Materials and Methods

5.1.2.1 Experimental set-up

This experiment was carried out over a two-week period in which LB agar plates were inoculated with 100 µL of concentrated Serenade ASO® diluted down $x10^{-8}$. 100 µL of Serenade ASO® diluted down $x10^{8}$ contains on average 10.8 CFU. Inoculated

plates were incubated at nine different temperature combinations. Temperatures were either kept constant or changed diurnally. Constant temperatures were 22°C, 15°C, 11°C and 4°C. Diurnal temperature combinations were 22°C & 4°C, 22°C & 11°C, 15°C & 4°C, 15°C & 11°C and 11°C & 4°C. Plates under the diurnal temperature combination regimes were kept at warmer day time temperatures for 8 h and at colder night time temperatures for 16 h. Four plates were incubated per temperature regime and plates were kept in the dark.

To assess the effect of temperature regime on the germination and growth rate of *B. amyloliquefaciens* QST 713, every 24 hours CFU counts were taken and a photograph of each plate was taken over a 2-week period. Photographs were analysed using ImageJ software to determine the area of the Petri dish covered by *B. amyloliquefaciens* colonies each day. Bacterial growth rate for each temperature regime could then be determined. Plates that exhibited no bacterial growth after two weeks were left for another three weeks at their respective incubation temperatures to see if growth occurred. Following this, plates were incubated at 27°C for 24 h before CFU counts were taken.

5.1.2.2 Statistical analysis

Colony area data was analysed via a repeated measurements ANOVA in Genstat 17.0. Microsoft Excel was used to calculate standard errors. A general ANOVA was used to analyse the differences in CFU counts between plates that were re-incubated at 27°C and to analyse the differences in average colony size.

5.1.3 Results

Table 5-1: The effect of temperature regime on the time taken to first spore germination and average CFU counts for Bacillus amyloliquefaciens *QST 713.*

Temperature regime	Time to first spore germination (h)	Average number of colony forming units per plate
22°C	40	17.5
15°C	144	5.5
22°C (8h) and 4°C (16h)	120	19
22°C (8h) and 11°C (16h)	120	15.8
4°C	No growth	0
11°C	No growth	0
11°C (8h) 4°C (16h)	No growth	0
15°C (8h) 4°C (16 h)	No growth	0
15°C (8h) 11°C (16h)	No growth	0

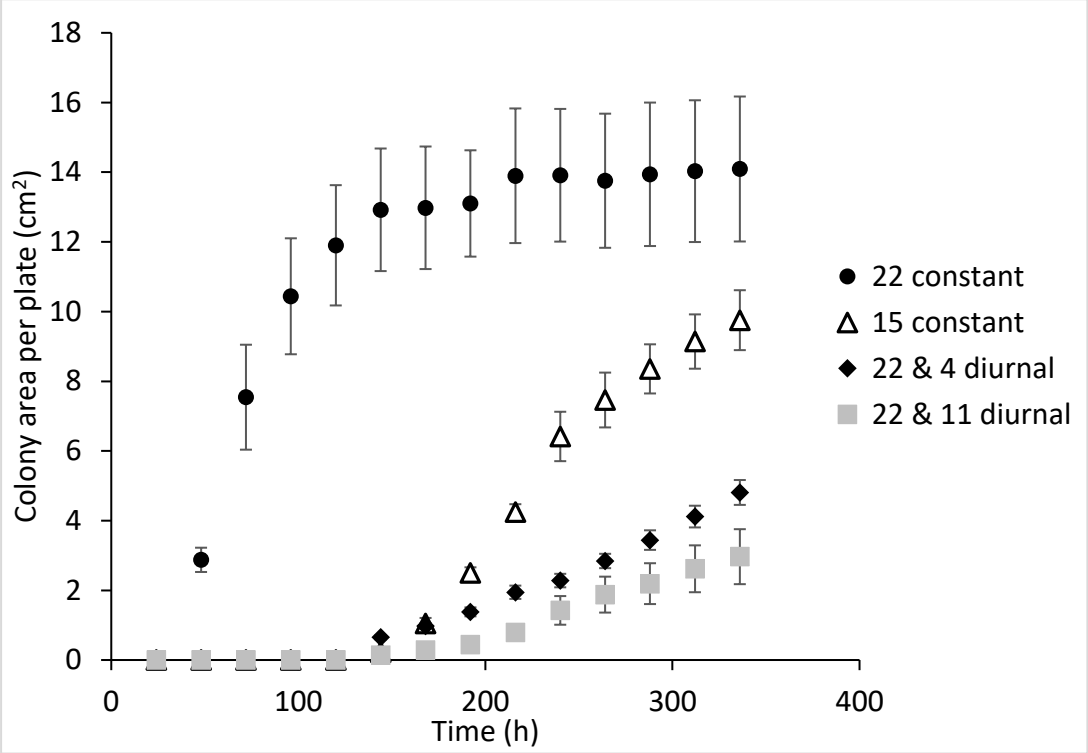

Figure 5-1: The effect of temperature regime (°C) on the colony area per plate of Bacillus amyloliquefaciens *QST 713 measured over two weeks. Standard error bars shown.*

The only temperature regimes which resulted in *B. amyloliquefaciens* growth *in vitro* were 22°C, 15°C, 22°C & 4°C and 22°C & 11°C (Table 5-1). Other temperature regimes (11°C & 4°C, 15°C & 4°C, 15°C & 11°C, 11°C constant and 4°C constant) resulted in no growth and were left incubating for another three weeks. Following this, still no *B. amyloliquefaciens* growth was seen, surprisingly not even under the diurnal temperature regime 15°C & 11°C. This suggests that under *in vitro* conditions, on LB nutrient agar, *B. amyloliquefaciens* spores do not germinate at temperatures below 15°C. For temperature regimes which did result in *B. amyloliquefaciens* growth, changing diurnal temperature appeared to affect both the

germination rate of *B. amyloliquefaciens* spores and the growth rate of bacterial colonies. Incubation at 22°C resulted in spore germination within 40 h, but when the night time temperature was reduced to both 4°C and 11°C spore germination did not occur until 120 h of incubation (Table 5-1). Spore germination took 144 h at an incubation temperature of 15°C, shown by the extended lag phase observed in Figure 5-1. The growth rate of *B. amyloliquefaciens* colonies (Figure 5-1) was higher at 22°C than with the lower temperature regimes. A significant interaction between time and the temperature regime was observed between the highest temperature regime, 22°C, and the lower temperature regimes, 15°C (P<0.001), 22&4°C (P=0.001) and 22&11°C (P<0.001). A significant interaction in time and temperature regime was also observed between 15°C and 22&4°C (P=0.001). Lower night time temperatures reduced the growth rate of colonies significantly.

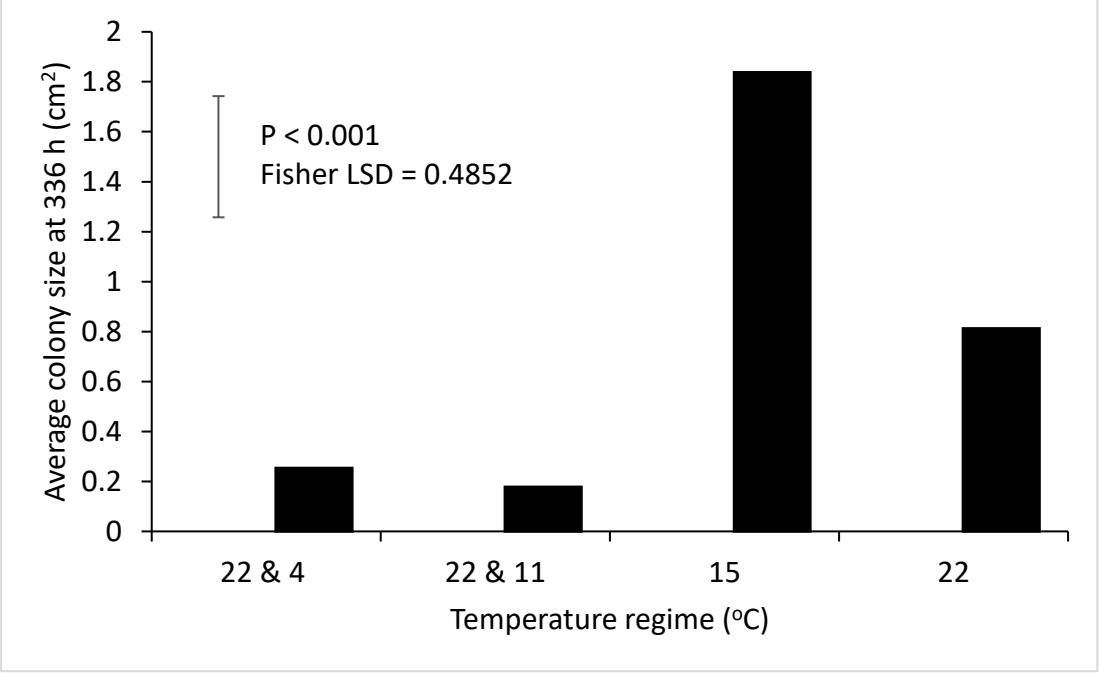

Figure 5-2: The effect of incubation temperature on the average size of Bacillus amyloliquefaciens *QST 713 colonies.*

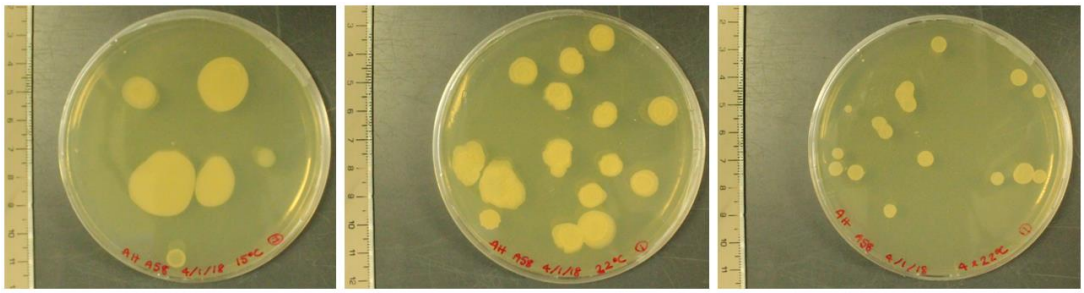

Figure 5-3: LB agar plates inoculated with Bacillus amyloliquefaciens *QST 713 (x10^{-8} Serenade ASO®) after 336h of incubation at 15°C (left), 22°C (middle) or 4&22°C (right).*

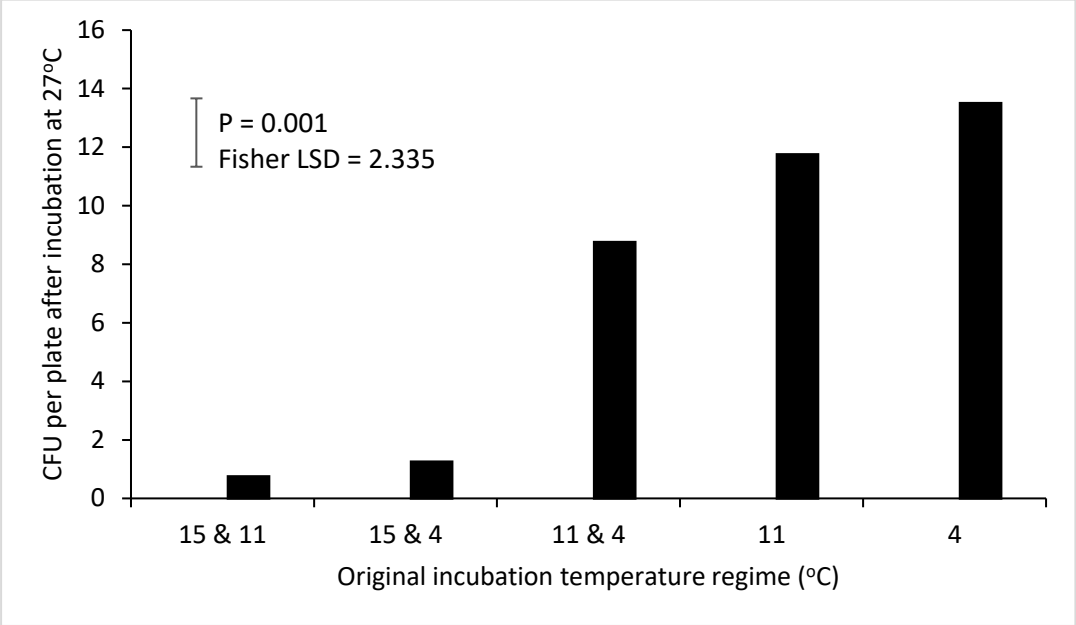

Figure 5-4: The effect of temperature regime on Bacillus amyloliquefaciens *QST 713 CFU count per plate after 5 weeks of incubation at their respective temperatures (which resulted in no bacterial growth) followed by 24 hours incubation at 27°C.*

As well as being slow to germinate, *B. amyloliquefaciens* incubated at 15°C exhibited CFU counts which were much reduced compared to the other treatments (Table 5-1). However, these fewer colonies then exhibited a rapid growth rate not dissimilar to the 22°C treatment growth rate. At 15°C average colony area was significantly larger than for colonies grown at the other temperatures (Figure 5-2). The reduced number and increased size of *B. amyloliquefaciens* colonies grown at 15°C can be

observed in Figure 5-3. A significant difference was also observed between the average size of colonies incubated at 22°C and 22&4°C (P<0.001).

Under the lower temperature regimes of 4°C and 11°C, *B. amyloliquefaciens* growth did not occur until plates were incubated at 27°C, when germination occurred in similar numbers to plates incubated initially at 22°C (Figure 5-4). However, when plates initially incubated at 15&4°C and 15&11°C were incubated at 27°C, CFU counts were dramatically reduced, with some plates exhibiting no growth at all.

5.2 Experiment 3b – Inoculated plants

The previous experiment found that temperatures below 15°C were unfavourable for growth of *B. amyloliquefaciens* QST 713 on agar plates, which may indicate that under lower temperatures, *B. amyloliquefaciens* root colonisation could be inhibited. Taking this into account, the following experiment was designed to grow carrots under both the warmer temperature regime of 20°C day/10°C night and the lower temperature regime of 15°C day/10°C night.

5.2.1 Sub-hypotheses

- Lower temperatures limit *B. amyloliquefaciens* colonisation of carrot roots.
- Temperature affects the ability of *B. amyloliquefaciens* to promote growth of 10 week old carrots.

5.2.2 Materials and Methods

5.2.2.1 Experimental set-up

This experiment consisted of two treatments, two temperature regimes and eight replicates. Two controlled environment growth rooms were used, one at the higher temperature of 20°C during the day and 10°C at night, the other at the lower temperature of 15°C in the day and 10°C at night, both with a 12-hour photoperiod.

Each growth room contained 16 columns. At sowing, four carrot seeds (cv. Nairobi F1) per column were placed onto the prepared seedbed, as described in 2.1.1.

Half of the columns under each temperature regime were left untreated and half were treated with *B. amyloliquefaciens* QST 713 via a pipette treatment. Serenade ASO® suspension was prepared as described in 4.2.2 and 325.8 µL was pipetted onto each carrot seedbed. All carrot seeds were covered by 5 mm of soil before being watered from above. Columns were arranged in a randomised design within both growth rooms and were watered daily for 10 weeks. The growth rooms were equipped with halogen bulb lighting at an intensity of approximately 27.1 $\mu mol.m^{-2}\ s^{-1}$ and 26.0 $\mu mol.m^{-2}\ s^{-1}$ at the soil surface, for the higher and lower temperature growth rooms, respectively. Soil temperature probes confirmed that the temperature within the columns in the low temperature growth room hit a maximum of 15.4°C and a minimum of 9.5°C at approximately 10 cm depth and a maximum of 14.4°C and minimum of 9.6°C at a depth of 30 cm. Soil temperature probes in the higher temperature growth room gave a maximum reading of 22°C and a minimum reading of 9.7°C at a depth of 10 cm, and a maximum reading of 19°C and minimum reading of 10°C at 30 cm depth. Ten LB agar plates inoculated with 100 µL of $x10^{-8}$ Serenade ASO® (5.1.2.1) were incubated in each growth room to determine if *in vitro* growth would occur.

5.2.2.2 Growth parameter assessment

After 10 weeks, carrots were harvested and measured (2.2.1). Columns were divided into sections at the depths 0-15 cm, 30-45 cm and 75-90 cm, which were kept for root quantification (2.2.2). The rest of the soil was discarded. Root sections were

scanned separately. However, in order to get a representation of the whole root system, measurements from each section were collated to give 'total' root system data (tap root not included). Carrot tap roots were washed separately and oven dried to calculate dry weight.

5.2.2.3 Nutrient analysis

The elements assessed via ICP-MS included the macronutrients P, K, Ca, Mg and S and the micronutrients B, Zn, Cu, Se and Mo. Due to small amounts of soil dust contamination in some samples, results for elements such as Fe, Al, Mn, V Cr, Co, As, Li, Cs and Be are not presented. These elements appear in larger quantities in the soil but lesser quantities in plant material and therefore elemental concentration results were skewed. Where elements are taken up in large quantities by plants, small amounts of soil dust contamination are unlikely to affect results. All data was checked for outliers using boxplots before being analysed. Due to the insufficient amount of plant material produced under the lower temperature regime, replicates were bulked together for nutrient analysis of both carrot shoots and tap root material. This left three untreated replicates, four treated tap root replicates and six treated shoot material replicates under the low temperature regime. Where bulking of samples occurred, the same samples were chosen from both plant parts to keep results consistent.

5.2.2.4 DNA analysis

Rhizospheric samples were taken from the tap root, from within the depths 0-15 cm (lateral roots off the main tap root), 30-45 cm and 75-90 cm (2.4.1). DNA extraction

and qPCR were undertaken (2.4), to determine the ability of *B. amyloliquefaciens* to colonise the carrot root system.

5.2.2.5 Statistical analysis

Failed germination of one treated replicate and three untreated replicates under the lower temperature regime meant missing values had to be used when analysing the data. All carrot growth parameter and nutrient content data was analysed using a Two-way ANOVA in GenStat 17.0. Due to the bulking together of samples for nutrient analysis, some replicates were left with further missing values to balance the experimental design.

5.2.3 Results

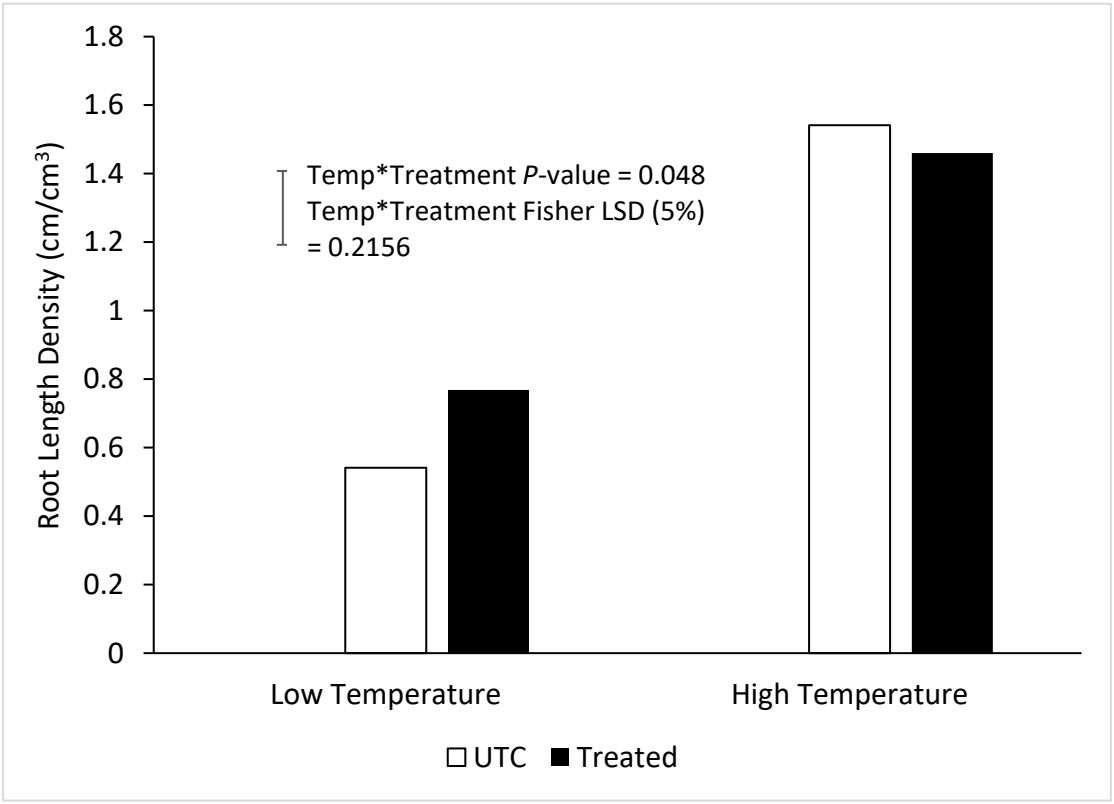

Figure 5-5: Root length density of 70-day old carrots under both high and low temperature regimes, treated with Bacillus amyloliquefaciens *QST 713.*

Table 5-2: The total root surface area of 70-day old carrots under both high and low temperature regimes, treated with Bacillus amyloliquefaciens *QST 713.*

Total Root Surface area (cm/cm^2)	**High Temperature**	**Low Temperature**	**Mean**
UTC	819	259	539
Treated	700	360	530
Mean	760	309	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	102.8	
Treatment	0.855	102.8	
Temperature.Treatment	0.037	145.3	

Under the high temperature regime there was no significant difference in carrot root length density between treatments (Figure 5-5). Contrary to this, under the lower temperature regime carrots treated with *B. amyloliquefaciens* had significantly increased root length density compared to control plants (*P*=0.048). This pattern was mirrored in total carrot root surface area, which also showed a significant interaction (*P*= 0.037) between treatment and temperature regime (Table 5-2). Under the high temperature regime there was a slight trend for increased root surface area in the untreated plants, whereas under the low temperature regime a significant trend was observed for *B. amyloliquefaciens* treatment increasing total root surface area. No

significant differences were observed between treatments in average root diameter at either temperature.

Other carrot growth parameters measured at harvest such as rooting depth, leaf area and shoot dry weight showed a similar pattern to the root data (Table 5-3), although no significant differences were observed. Although insignificant, a pattern was also observed in total root dry weight (which excluded the main tap root at 0-15 cm), where again under the low temperature regime root biomass was increased in treated plants, whereas under higher temperature conditions this pattern was reversed. No significant differences were observed in tap root dry weight, but Figure 5-6 suggests that there may have been some visual differences in tap root appearance following *B. amyloliquefaciens* treatment. Treated carrots (left), under both high (top) and low (bottom) temperature regimes appear to be brighter orange in colour and appear to have a larger circumference down the length of the tap root. In general, visually the treated carrots appear healthier and more developed at this stage.

Overall, carrot growth parameter measurements suggest that at 70 das, under warmer temperatures such as 20^{o}C/10^{o}C, there were no obvious benefits of treatment with *B. amyloliquefaciens* QST 713. However, when temperatures were reduced to 15^{o}C/10^{o}C, benefits to plant growth were observed, more specifically through increased root length density.

Table 5-3: Above and below ground growth parameters of 70-day old carrots, under both high and low temperature regimes, untreated (UTC) or treated with Bacillus amyloliquefaciens *QST 713.*

Root Depth (cm)	**High Temperature**	**Low Temperature**	**Mean**
UTC	109.6	98.2	103.9
Treated	105.9	102.9	104.4
Mean	107.8	100.5	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	0.022	6.08	
Treatment	0.880	6.08	
Temperature.Treatment	0.167	8.60	

Shoot height (cm)	**High Temperature**	**Low Temperature**	**Mean**
UTC	23.8	11.6	17.7
Treated	24.5	13.6	19.1
Mean	24.2	12.6	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	2.00	
Treatment	0.171	2.00	
Temperature.Treatment	0.491	2.82	

Leaf Area (cm^2)	**High Temperature**	**Low Temperature**	**Mean**
UTC	173.4	41	107.2
Treated	164.4	61	112.7
Mean	168.9	51	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	18.64	
Treatment	0.551	18.64	
Temperature.Treatment	0.121	26.36	

Shoot dry weight (g)	**High Temperature**	**Low Temperature**	**Mean**
UTC	1.534	0.344	0.939
Treated	1.466	0.484	0.975
Mean	1.500	0.414	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.1823	
Treatment	0.684	0.1823	
Temperature.Treatment	0.251	0.2578	

Carrot Tap Root Dry Weight (g)	High Temperature	Low Temperature	Mean
UTC	0.746	0.173	0.459
Treated	0.775	0.206	0.491
Mean	0.761	0.190	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.1445	
Treatment	0.659	0.1445	
Temperature.Treatment	0.977	0.2044	

Total Root Dry Weight (g)	High Temperature	Low Temperature	Mean
UTC	0.2034	0.0686	0.1360
Treated	0.1847	0.0942	0.1395
Mean	0.1941	0.0814	
	***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.0249	
Treatment	0.779	0.0249	
Temperature.Treatment	0.079	0.0352	

Figure 5-6: Bacillus amyloliquefaciens *QST 713 treated (T) and untreated (U) carrot tap roots after 70-days of growth under high (H) and low (L) temperature regimes.*

Table 5-4: Macro- and micronutrient content[5] of carrot tap roots grown for 70 days under high and low temperature regimes, untreated (UTC) or treated with Bacillus amyloliquefaciens *QST 713.*

P (mg)	**High**	**Low**	**Mean**	**S (mg)**	**High**	**Low**	**Mean**
UTC	3.23	0.61	1.92	**UTC**	1.154	0.259	0.706
Treated	3.22	0.76	1.99	**Treated**	1.352	0.281	0.816
Mean	3.22	0.69		**Mean**	1.253	0.270	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.777		**Temperature**	<.001	0.325	
Treatment	0.847	0.777		**Treatment**	0.487	0.325	
Temperature. Treatment	0.838	1.099		**Temperature . Treatment**	0.576	0.460	

K (mg)	**High**	**Low**	**Mean**	**Ca (mg)**	**High**	**Low**	**Mean**
UTC	29.4	6.9	18.2	**UTC**	3.61	0.94	2.27
Treated	29.5	8.4	19.0	**Treated**	3.56	1.17	2.37
Mean	29.5	7.6		**Mean**	3.58	1.06	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	6.17		**Temperature**	<.001	0.667	
Treatment	0.789	6.17		**Treatment**	0.778	0.667	
Temperature. Treatment	0.834	8.72		**Temperature . Treatment**	0.659	0.944	

Mg (mg)	**High**	**Low**	**Mean**	**B (mg)**	**High**	**Low**	**Mean**
UTC	1.077	0.329	0.703	**UTC**	0.0324	0.0059	0.0191
Treated	1.030	0.350	0.690	**Treated**	0.0340	0.0073	0.0206
Mean	1.053	0.340		**Mean**	0.033	0.007	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.195		**Temperature**	<.001	0.007	
Treatment	0.891	0.195		**Treatment**	0.653	0.007	
Temperature. Treatment	0.719	0.276		**Temperature . Treatment**	0.972	0.010	

[5] Nutrient content is a product of nutrient concentration (mg/kg) and shoot dry weight (kg)

Cu (mg)	High	Low	Mean	Zn (mg)	High	Low	Mean
UTC	0.0072	0.0016	0.0044	**UTC**	0.0216	0.0054	0.0135
Treated	0.0065	0.0020	0.0042	**Treated**	0.0220	0.0064	0.0142
Mean	0.0069	0.0018		**Mean**	0.0218	0.0059	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.0014		**Temperature**	<.001	0.005	
Treatment	0.804	0.0014		**Treatment**	0.777	0.005	
Temperature. Treatment	0.441	0.0020		**Temperature . Treatment**	0.913	0.007	

Se (mg)	**High**	**Low**	**Mean**	**Mo (mg)**	**High**	**Low**	**Mean**
UTC	5.84E-05	1.71E-05	3.77E-05	**UTC**	2.02E-04	4.41E-05	1.23E-04
Treated	5.83E-05	2.12E-05	3.97E-05	**Treated**	2.37E-04	5.73E-05	1.47E-04
Mean	5.83E-05	1.91E-05		**Mean**	2.20E-04	5.07E-05	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	1.13E-05		**Temperature**	<.001	3.51E-05	
Treatment	0.716	1.13E-05		**Treatment**	0.166	3.51E-05	
Temperature. Treatment	0.707	1.60E-05		**Temperature . Treatment**	0.522	4.96E-05	

Table 5-5: Macro- and micronutrient content[6] of carrot shoots after 70 days of growth under high and low temperature regimes, untreated (UTC) or treated with Bacillus amyloliquefaciens *QST 713.*

P (mg)	**High**	**Low**	**Mean**	**S (mg)**	**High**	**Low**	**Mean**
UTC	4.05	1.12	2.58	**UTC**	6.38	1.51	3.94
Treated	3.95	1.41	2.68	**Treated**	6.32	2.03	4.18
Mean	4.00	1.26		**Mean**	6.35	1.77	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.536		**Temperature**	<.001	1.02	
Treatment	0.703	0.536		**Treatment**	0.639	1.02	
Temperature. Treatment	0.462	0.758		**Temperature. Treatment**	0.564	1.44	

[6] Nutrient content is a product of nutrient concentration (mg/kg) and shoot dry weight (kg)

K (mg)	High	Low	Mean	Ca (mg)	High	Low	Mean
UTC	51.8	11.3	31.6	**UTC**	43.0	9.3	26.2
Treated	51.4	14.9	33.1	**Treated**	41.6	14.0	27.8
Mean	51.6	13.1		**Mean**	42.3	11.7	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	7.21		**Temperature**	<.001	5.34	
Treatment	0.659	7.21		**Treatment**	0.528	5.34	
Temperature. Treatment	0.566	10.19		**Temperature. Treatment**	0.258	7.56	

Mg (mg)	**High**	**Low**	**Mean**	**B (mg)**	**High**	**Low**	**Mean**
UTC	4.40	0.81	2.60	**UTC**	0.0661	0.0130	0.0395
Treated	3.65	1.21	2.43	**Treated**	0.0659	0.0181	0.0420
Mean	4.03	1.01		**Mean**	0.0660	0.0155	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.634		**Temperature**	<.001	0.011	
Treatment	0.575	0.634		**Treatment**	0.635	0.011	
Temperature. Treatment	0.074	0.896		**Temperature. Treatment**	0.608	0.015	

Cu (mg)	**High**	**Low**	**Mean**	**Zn (mg)**	**High**	**Low**	**Mean**
UTC	0.012	0.003	0.008	**UTC**	0.0316	0.0090	0.0203
Treated	0.011	0.004	0.008	**Treated**	0.0309	0.0096	0.0203
Mean	0.012	0.004		**Mean**	0.0313	0.0093	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	0.0014		**Temperature**	<.001	0.005	
Treatment	0.846	0.0014		**Treatment**	0.968	0.005	
Temperature. Treatment	0.231	0.0020		**Temperature. Treatment**	0.765	0.007	

Se (mg)	**High**	**Low**	**Mean**	**Mo (mg)**	**High**	**Low**	**Mean**
UTC	1.09E-04	3.40E-05	7.14E-05	**UTC**	0.00213	0.00051	0.00132
Treated	9.86E-05	4.04E-05	6.95E-05	**Treated**	0.00206	0.00052	0.00129
Mean	1.04E-04	3.72E-05		**Mean**	0.00209	0.00051	
	***P*-value**	**Fisher LSD (5%)**			***P*-value**	**Fisher LSD (5%)**	
Temperature	<.001	1.84E-05		**Temperature**	<.001	0.0002	
Treatment	0.834	1.84E-05		**Treatment**	0.753	0.0002	
Temperature. Treatment	0.361	2.61E-05		**Temperature. Treatment**	0.694	0.0003	

No significant differences were observed in the macro- and micronutrient concentration (mg/kg) of the carrot shoots and tap roots (results not shown), but when the total nutrient content (mg) of both shoots and tap roots were calculated (results in Table 5-4 and Table 5-5), although not significant, a slight trend could be observed, mirroring the trend seen in carrot growth parameters, in particular root length density.

Table 5-6: Average Bacillus amyloliquefaciens *CFU g^{-1} detected in the rhizosphere of 70 day old carrots at various depths following growth under high and low temperature regimes.*[7] *The number of positive reactions is out of a total of three qPCR reactions per replicate.*

Temperature	Treatment	Depth	Rep	Average CFU g^{-1}	No. positive reactions
High	Control	0-15	1	ND	0
			2	ND	0
			3	ND	0
			4	ND	0
			5	ND	0
			6	ND	0
			7	ND	0
			8	ND	0
High	Treated	0-15	2	1.34E+05	2
			3	1.98E+05	1
			4	5.74E+04	2
			5	8.61E+05	3
			6	8.54E+05	3
			7	3.61E+05	3
			8	9.99E+05	2
High	Treated	30-45	1	4.28E+05	2
			2	ND	0
			3	ND	0
			4	ND	0
			5	ND	0
			6	ND	0
			7	ND	0
			8	ND	0
High	Treated	75-90	1	ND	0
			2	ND	0
			3	ND	0
			4	ND	0
			5	ND	0
			6	ND	0
			7	ND	0
			8	ND	0
High	Treated	Bottom	1	ND	0
			2	ND	0
			3	ND	0
			4	ND	0
			5	ND	0
			6	ND	0
			7	ND	0
			8	ND	0

[7] *Bacillus subtilis* CFU g^{-1} values presented here are the average of the CFU values for each replicate (not including ND results). ND results were determined by a Cq value >35 and/or a Tm value outside the range of 78 +/- 1°C.

Temperature	Treatment	Depth	Rep	Average CFU g^{-1}	No. positive reactions
Low	Control	0-15	1	ND	0
			3	ND	0
			5	ND	0
			6	ND	0
			8	ND	0
Low	Treated	0-15	1	6.35E+05	3
			2	3.12E+05	1
			4	5.64E+05	3
			5	1.60E+05	3
			6	1.72E+05	1
			7	2.08E+05	3
			8	1.39E+06	6
Low	Treated	30-45	1	ND	0
			2	3.13E+04	1
			4	ND	0
			5	ND	0
			6	ND	0
			7	ND	0
			8	ND	0
Low	Treated	75-90	1	ND	0
			2	ND	0
			4	ND	0
			5	ND	0
			6	2.84E+04	1
			7	ND	0
			8	ND	0
Low	Treated	Bottom	1	2.07E+04	1
			2	ND	0
			4	ND	0
			5	ND	0
			6	9.16E+04	2
			7	ND	0
			8	ND	0

Results from qPCR analysis showed that *B. amyloliquefaciens* QST 713 was able to colonise the roots of carrots grown under both the high and low temperature regimes (Table 5-6) (raw data, Table 8-4). However, under the high temperature regime, *B. amyloliquefaciens* was only found in the top 0-15 cm of the root system whereas under the lower temperature regime *B. amyloliquefaciens* was detected

right down the tap root. *B. amyloliquefaciens* was found in high densities of approximately 10^5 CFU g^{-1}, at the depth 0-15 cm under both temperature regimes. Under the low temperature regime *B. amyloliquefaciens* density and detection rates gradually decreased further down the root system. Multiple samples from both temperature regimes detected *B. amyloliquefaciens* at lower levels (10^3 CFU g^{-1}), however due to the Cq value exceeding 35, these results were not reliable. Low levels of *B. amyloliquefaciens* were also detected in some control rhizosphere samples for both the high and low temperature regimes, which suggests contamination, but these results were also not reliable. Control samples were re-run through qPCR to check that contamination did not occur while setting up the assay, but again levels at 10^3 CFU g^{-1} were detected. Contamination therefore must have occurred either during DNA extraction or during carrot growth.

Interestingly, all the LB agar plates inoculated with *B. amyloliquefaciens* QST 713 exhibited bacterial growth when incubated in the growth rooms under both temperature regimes (20°C & 10°C and 15°C & 10°C).

5.3 Discussion

Temperature is an important factor to consider when assessing the growth, survival and effectiveness of PGPR such as *B. amyloliquefaciens* in the rhizosphere (Budde et al., 2006, Pertot et al., 2013). Quantifying the population of *B. amyloliquefaciens* QST 713 in the rhizosphere is also important in order to understand how environmental conditions affect colonisation and determine the plant growth promotion effects associated with root colonisation (Mendis et al., 2018). These experiments assessed

the ability of *B. amyloliquefaciens* QST 713 to grow, both *in vitro* and *in vivo,* and promote carrot growth under low temperature conditions.

5.3.1 Experiment 3a – Growth on agar plates

In vitro, *B. amyloliquefaciens* QST 713 spores were seen to germinate and grow successfully at temperatures above and including the temperature regime of 15^{o}C constant. Of the temperature regimes which did result in germination, 22^{o}C resulted in the fastest germination and growth rate. The lower temperature regimes and colder diurnal regimes, such as 15^{o}C, 22/4^{o}C and 22/11^{o}C, caused a delay in *B. amyloliquefaciens* growth, dramatically increasing the time taken to first spore germination and elongating the lag phase of growth. These results are in line with a study by Pertot et al. (2013) who found that *in vitro*, temperatures below 30^{o}C caused a significantly elongated lag phase and slower growth rate of *B. amyloliquefaciens* S499 compared to temperatures above 30^{o}C. The study also found that *in vitro*, lower temperatures caused strains S449, FBZ42 and QST 713 to be less motile with reduced swarming abilities. These results might be explained by Budde et al. (2006), who suggest rapid temperature change or extended growth at particularly high or low temperatures can induce adaptive responses within *B. subtilis* cells, causing repression in gene expression resulting in slower growth rates. The study found expression of genes involved in *B. subtilis* motility, chemotaxis and metabolism to be among those affected by colder temperatures.

Of particular interest in these results was the effect that the 15^{o}C temperature regime had on the germination and growth of *B. amyloliquefaciens* QST 713 *in vitro.*

CFU counts for the 15°C regime were dramatically lower than for the other temperatures, suggesting successful germination was challenging at this temperature. *B. amyloliquefaciens* CFU per plate at 15°C was 5.5 CFU compared to 17.5 CFU at 22°C. The average colony size on plates grown at 15°C, however, was significantly larger, suggesting that once germination of the reduced number of spores occurred, swarming and colony growth were unaffected at 15°C and the colonies grew to a similar size, if not larger, as those grown at 22°C. This contradicts studies by Pertot et al. (2013) and Budde et al. (2006), who suggest that at lower temperatures, reduced swarming ability and motility of *Bacillus* species, including QST 713, should cause a reduction in colony diameter compared to those grown at warmer temperatures. One potential explanation for this finding could be that the reduced colony numbers on 15°C agar plates resulted in a larger supply of nutrients for colony expansion compared to other more overcrowded plates which may have been limited by nutrient availability. Pertot et al. (2013) also found *in vitro* surfactin production in *Bacillus* strains S499 and FBZ42 to be much higher within the temperature range 15-20°C than at 30°C and above. This might explain the lack of colony size reduction at 15°C, as surfactin production is an important contributor to the ability of *Bacillus* species to swarm (Kearns, 2010, Ongena and Jacques, 2008). However, temperature was not found to have such a strong effect on the *in vitro* surfactin production of QST 713 and so no clear conclusion can be made.

When plates that exhibited no growth after five weeks were incubated at 27°C, germination rates were high for the lower temperature regimes 4°C, 11°C and 11/4°C, but significantly lower for temperature regimes including 15/4°C and

15/11°C. It could be that at 15°C spores begin to germinate slowly but before they can fully germinate they are checked by the colder night time temperature, resulting in unsuccessful germination. This night time temperature check may be critical, as significantly slower growth was seen under the temperature regimes 22/4°C and 22/11°C. However, here it is possible that the day time temperature of 22°C was enough for spores to germinate fully before being checked, unlike at 15°C. Interestingly, the temperature at which *B. amyloliquefaciens* germination becomes limited in this experiment, 15°C, corresponds to the temperature which researchers reduce *B. subtilis* to rapidly during *in vitro* experiments, in order to elicit a cold shock response (Budde et al., 2006). This could begin to explain the difficulty in germination observed at temperatures below 15°C. However, Budde et al. (2006) also discuss that gradual changes in the soil temperature are not enough to elicit cold shock responses. Instead *B. subtilis* is able to alter its gene expression to adapt to growth at colder temperatures, known as chill adaptation. Where the lower temperatures affected *B. amyloliquefaciens* growth *in vitro* here, the insulating effect of the soil and gradual temperature changes may mean that *B. amyloliquefaciens* is therefore able to adapt *in vivo*.

Results from the *in vitro* experiment indicate that *B. amyloliquefaciens* QST 713 growth *in vitro* was not favourable at temperatures below 15°C. *Bacillus amyloliquefaciens* growth did occur *in vitro* when night temperatures were as low as 4°C, however the day temperature for this treatment was 22°C, allowing rapid germination and growth. Schmidt et al. (2004) found that day time temperature, irrespective of the night time temperature drop, had an impact on the biocontrol

efficacy of *Pseudomonas fluorescens,* suggesting that the day time temperature exposure is an important factor for PGPR. However, in the same study diurnal temperature regime was not seen to affect *B. subtilis* MBI 600 populations.

Interestingly, growth at 15/11^{o}C was not observed *in vitro* in Experiment 3a, but when similar plates were incubated in the growth rooms used for Experiment 3b, bacterial growth was observed at 15/10^{o}C at a rate similar to the plates grown at 20/10^{o}C. These conflicting results could be due to the impact of the differing durations of exposure to high and low temperature as Experiment 3a had 8h at high temperature while 3b had 12h. Kloepper et al. (2007) found that a reduction in photoperiod lessened the early colonisation rate and the growth promotion ability of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a. This could begin to explain why there was a difference in QST 713 growth between the experiments. However, in our experiments plates were kept in the dark and so the effect of light was not a factor. Instead it may be the increased exposure to the day time temperature of 15^{o}C that allowed bacterial growth *in vitro* (Schmidt et al., 2004). Another potential explanation for these results could be the differing incubation conditions between the two experiments. In Experiment 3a plates were kept in dark, temperature regulated incubators, whereas in Experiment 3b plates were wrapped in black card and kept on a bench in the growth rooms underneath the lighting rig. It is possible that the lights may have heated the black card during the day resulting in a slight increase in temperature. For a true comparison of changing day time temperature length, the same incubators should have been used.

Following the results from the *in vitro* experiment, where the diurnal temperature combination 15/11°C appeared to inhibit *B. amyloliquefaciens* germination and growth, we decided to use 15/10°C as the lower temperature regime in Experiment 3b. The experiment was originally designed to use 20/15°C as the warmer temperature regime, but due to a technical fault the warmer growth room also experienced a night time temperature of 10°C.

5.3.2 Experiment 3b – Inoculated plants

Carrots were grown for 70 days to assess whether treatment with *B. amyloliquefaciens* QST 713 would promote growth at this stage. It was hypothesized that, if growth promotion was indeed evident at this stage then growing carrots under the lower temperature regime would affect the ability of *B. amyloliquefaciens* QST 713 to colonise roots and in turn the ability of the bacteria to promote growth. This, however, was not the case: no growth promotion was observed under the warmer temperature regime, and instead under the lower temperature regime the root length density of treated carrots was significantly larger than that of untreated carrots. This trend was also observed in carrot root surface area and although not statistically significant, in leaf area, shoot dry weight and rooting depth. Although not statistically significant, a slight trend was observed for *B. amyloliquefaciens* treatment causing an increase in shoot height under both temperature regimes.

The trend observed in carrot nutrient uptake under the various treatments, although not statistically significant, seemed to mirror the effect that *B. amyloliquefaciens* treatment had on carrot root length density. Under the lower temperature regime

there appeared to be a trend for increased tap root nutrient uptake in treated plants compared to UTC plants (Table 5-4). Under the higher temperature regime, no significant differences between UTC and treated plants or trends were observed. Nutrient content results for carrot shoots (

Table 5-5) showed that under the high temperature regime a slight trend for untreated shoots having a marginally higher nutrient content was observed, whereas under the lower temperature regime this trend was reversed, and treated plants appeared to have increased nutrient uptake. The relationship between the increased carrot root development and nutrient uptake is in line with a study by Klopper et al. (2007), who suggest that increasing the surface area of the root system increases the exploration of the soil, therefore directly affecting nutrient uptake. The increase in root length density observed in this experiment under the lower temperature regime could therefore explain the slight increase in carrot nutrient content.

Overall, growth promotion of 70-day old carrots by *B. amyloliquefaciens* QST 713 was not observed under the warmer temperature regime, whereas when plants were subject to colder temperatures and therefore temperature stress, treatment with *B. amyloliquefaciens* QST 713 appeared to have a significant effect on carrot growth, particularly on root development. Treatment with *B. amyloliquefaciens* QST 713 therefore appeared to alleviate low temperature stress, even though under normal conditions a growth promotion effect was not observed. Although not a *Bacillus* species, another PGPR, *Serratia nematodiphila* PEJ1011, was found to promote growth of pepper plants while alleviating the effects of low temperature

stress caused by exposing plants to four hours of cold shock at 5°C (Kang et al., 2015). The ability of *S. nematodiphila* to alleviate temperature stress was thought to be due to regulation of phytohormones such as endogenous gibberellin and abscisic acid, jasmonic and salicylic acid. Growth promotion in the study by Kang et al. (2015) was also observed under warmer temperature conditions (28/25°C) and so their results do not shed light on why growth promotion in our experiment was seen under the lower temperature regime but not under the higher temperature regime. Although not a PGPR effect, a study by Helbig (2001) also found that at 10°C, *Paenibacillus polymyxa* was able to suppress *Botrytis cinerea* infection on strawberry leaf discs effectively, indicating that *Bacillus* colonisation and successful biocontrol do occur at low temperatures such as those seen in the field.

In a review by Brown (1974), it is stated that microbial inoculation of the seed will benefit vegetable crops such as carrots as an increased leaf area will increase photosynthetic rate and therefore tap root size. However, in this experiment, even though leaf area was slightly increased by *B. amyloliquefaciens* treatment under the low temperature regime at the 70-day stage, no increase in tap root dry weight was observed. A slight visual difference in carrot tap root appearance was observed between treatments, as under the warmer temperature regime treated carrots appeared to be brighter orange in colour with a slightly increased diameter down the tap root, and under the lower temperature regime treated carrot tap roots appeared slightly larger. It might have been interesting to scan the tap roots with WinRHIZO, as differences in diameter might have been detected between the treatments. The greatest growth promotion effects observed by *B. amyloliquefaciens* QST 713 were

on carrot root development, but carrot tap roots were not included in these measurements. Only three 15 cm depths of the carrot root system were washed and analysed via WinRHIZO at different points down the column. This was due to the time required to wash and scan the roots before they began to break down. Assessing root data is time consuming and therefore some researchers use gnotobiotic or hydroponic methods (Fan et al., 2012, Pertot et al., 2013, Simons et al., 1996) to remove the soil element from experiments. However, this means results are not representative of field conditions and there is much reduced competition from other rhizosphere dwelling microorganisms (Kloepper and Beauchamp, 1992). Using soil in this experiment was therefore thought necessary, although it meant only a representative proportion of the roots was quantified, not the whole root system.

qPCR results revealed that the lower temperature regime did not appear to reduce the ability of *B. amyloliquefaciens* to colonise the carrot root system at any depth. This is in line with the study by Pertot et al. (2013), who found *B. amyloliquefaciens* S499 able to colonise bean, tomato and zucchini roots at 15^{o}C, with no significant differences observed in the colonisation ability of the strain between the temperatures 15^{o}C and 35^{o}C. In agreement, Schmidt et al. (2004) found temperature to have no effect on the population of *B. subtilis* MBI 600 spores recovered from sugar beet seedling roots. Spores were recovered across a temperature range of 3-35^{o}C with the diurnal temperatures tested including 3/10^{o}C, 10/20^{o}C and 3/25^{o}C. This study could suggest that the *B. amyloliquefaciens* DNA we detected is *B. amyloliquefaciens* in spore form, but the density of DNA detected on

the small areas of root we sampled compared to the number of *B. amyloliquefaciens* CFU added to each column indicate that the spores are germinating and multiplying.

Bacillus amyloliquefaciens QST 713 colonised 70 day old carrot roots under both high and low temperature regimes but to varying degrees. Detection rates in the top 0-15 cm were high for both temperatures at 10^5 CFU g^{-1}, but under the warmer temperature regime colonisation did not occur further down the tap root, unlike in the lower temperature regime where colonisation could be observed at 10^4 CFU g^{-1} down the tap root to the root tip. Comparable *B. amyloliquefaciens* QST 713 detection rates of 10^4-10^5 CFU g^{-1} of root were observed on maize roots in a very similar experiment by Mendis et al. (2018) lasting 28 days. Similarly, *Bacillus* strains GB03 and GB99 were recovered from the rhizosphere of *Capsicum annum* in field trials at a rate of 10^3 and 10^4 CFU g^{-1} of root respectively (Kokalis-Burelle et al., 2006). It appeared that colonisation at 15/10^{o}C was improved at depth compared to 20/10^{o}C and this trend was also seen in the study by Schmidt et al. (2004), who found the temperature combination 20/10^{o}C to increase the *B. subtilis* colonisation density further down the sugar beet seedling root system compared to other temperature combinations such as 10/3^{o}C and 25/3^{o}C. The study by Pertot et al. (2013) may offer a possible explanation for this observation.

Despite finding no differences in the ability of *B*. *amyloliquefaiens* S499 to colonise plant roots within the temperature range of 15-35^{o}C, Pertot et al. (2013) found there was a trend for increased ISR caused by the strain at 15^{o}C and 35^{o}C compared to 25^{o}C. This trend was mirrored in surfactin production by S499 in the rhizosphere of both tomatoes and beans grown under hydroponic conditions. Surfactin production

in the zucchini rhizosphere was found highest at 15°C, decreasing as temperature increased. Under gnotobiotic conditions, surfactin was produced by S499 on the rhizosphere of tomato plantlets in much larger quantities at 15°C, approximately 1800 µg 10^8 CFU^{-1}, than at the temperatures 25°C and 35°C which were 16 and 4 µg 10^8 CFU^{-1}, respectively. This increased production of surfactin at lower temperatures could begin to explain why *B. amyloliquefaciens* QST 713 colonisation under the lower temperature regime was greater than under the higher temperature regime and why a slight growth promotion effect was only observed under the lower temperature regime of 10-week old carrots. Pertot et al. (2013) do highlight, however, that these results cannot be true of all similar *B. amyloliquefaciens* strains. Therefore, further investigation into the secondary metabolite production of strain QST 713 must be carried out in order to come to a conclusion, particularly as *in vitro*, Pertot et al. (2013) found surfactin production by strain QST 713 not to be significantly affected by temperature, in contrast to strains S499 and FZB42.

Pertot et al. (2013) also found that growth at 15°C resulted in biofilm formation *in vitro* equivalent to or even enhanced over, that formed at 30°C. Formation of biofilms results in bacterial cells becoming less sensitive to low temperature fluctuations (Perrot et al., 1998) and this was thought to explain the similar colonisation abilities of *B. amyloliquefaciens* seen at higher and lower temperatures by Pertot et al. (2013). Pertot et al. (2013) suggest that slow bacterial growth (as observed in Exp. 3a due to lower temperatures) may influence higher surfactin production in the rhizosphere, particularly under lower nutrient levels. As surfactin production is thought to be an important factor for biofilm formation and stable root

colonisation (Bais et al., 2004, Ben Abdallah et al., 2018), this might suggest that under the lower temperature regime biofilm production was enhanced and therefore root colonisation was improved compared to that at the higher temperature.

Bacillus amyloliquefaciens was detected further down the root system of carrots under the warmer temperature regime, but levels of detection were lower than the accurate detection limit of the assay (Cq>35) and so they are not reliable. Borriss (2011) also reports the minimum bacterial population to cause a plant growth promotion response to be 10^4 CFU g^{-1} of root. The lack of detectable root colonisation further down the tap root could begin to explain why no plant growth promotion was observed under the warmer temperature regime. It may be that the slower growth rate of the carrots under the cooler regime allowed for colonisation down the root system and under the higher temperature regime root growth may have exceeded the spreading ability of *B. amyloliquefaciens* (Fan et al., 2012).

Finally, Pertot et al. (2013) discuss how *B. amyloliquefaciens* is able to alleviate temperature stress, resulting in ISR benefits. Although very different, the concept could be transferred to this experiment and suggest that under lower temperature stress conditions QST 713 is able to promote growth slightly to alleviate stress. Ramalingam et al. (2017) discusses the ability of *Bacillus* species to alleviate other abiotic stresses such as drought, salinity and heavy metals.

An improvement to this experiment would be to pre-germinate the carrot seeds before sowing as this would help to prevent failed or slow emergence. It is also worth mentioning that the experimental design for this experiment was not optimal

as the number of growth rooms was a limiting factor. In ideal circumstances we would have used multiple growth rooms for each temperature to account for any differences between the rooms. A further improvement to the 70 day carrot experiment would have been to increase the number of temperature regimes, with samples taken at different growth stages. However, due to the destructive processes involved in sampling the roots, too many replicates and growth rooms would have been required.

The outcome from these experiments was unexpected. The effect that temperatures of 15^{o}C and below had on the growth of *B. amyloliquefaciens* QST 713 *in vitro* indicated that low temperatures may also affect root colonisation. Our results, however, showed that under low temperatures *B. amyloliquefaciens* root colonisation was instead improved and a growth promotion effect was observed. Again unexpectedly, no growth promotion effect was seen in carrots grown under normal temperature conditions after 70 days. These results indicate the potential for *B. amyloliquefaciens* QST 713 to be used in alleviating low temperature stress in crops. Further work should be carried out to investigate this more thoroughly as although studies are abundant on the impacts of *Bacillus* species on drought, salinity and heavy metal stress (Radhakrishnan et al., 2017), studies on *Bacillus* species for amelioration of low temperature stress are limited.

Chapter 6 General Discussion

Investigation into the effective use of biostimulants in agriculture is becoming increasingly important due to the rising global population, the changing climate, the decreasing number of effective chemical pesticide options and the need for sustainable crop management (Chandler et al., 2008, Gouda et al., 2018). In light of this, our study had multiple aims. The first was to determine whether root colonisation by *B. amyloliquefaciens* QST 713 resulted in growth promotion of carrot and potato crops, in turn leading to increased nutrient uptake. The second aim was to determine the application method for the bacterial inoculant which leads to the most efficient root colonisation and if observed, growth promotion effect. The final aim was to assess the effect of low soil temperature on the ability of *B. amyloliquefaciens* QST 713 to act as a biostimulant.

In order to address our first aim, controlled environment experiments were carried out on potatoes and carrots grown for various time periods (Chapters 3 &4). Results indicating the ability of *B. amyloliquefaciens* QST 713 to promote growth of the two crops differed hugely, despite the similar ability of the bacteria to colonise both root systems effectively. Colonisation of potato roots by *B. amyloliquefaciens* showed promising growth promotion effects, with significant increases in shoot height and root surface area observed. As well as the increase in root system development, nutrient uptake in potato shoots was also increased and it might be suggested that the change in root architecture caused the root system to explore a larger area of the soil leading to increased nutrient uptake (Kloepper et al., 2007). The results we observed here agree with other studies reporting the growth promotion effects of

Bacillus strain QST 713 on multiple food crops including wheat, maize, radish and cucumber (Accinelli et al., 2018, García-López et al., 2016, Garcia-Lopez et al., 2018, Meng et al., 2016). In contrast, under similar growth conditions, *B. amyloliquefaciens* QST 713 was not seen to promote growth of carrots after 21 days, 37 days or 70 days of growth. Considering the results observed in the potato experiment and in other studies this was surprising and a lack of literature regarding growth promoting effects by QST 713 on carrots makes it difficult to explain. One explanation for the lack of growth promotion may have been the application method and frequency at which *B. amyloliquefaciens* QST 713 was applied. As discussed in Chapter 4, many other studies observing successful growth promotion of crop plants applied *B. amyloliquefaciens* as a root drench (Ben Abdallah et al., 2018, Meng et al., 2016), often after sowing, and often used multiple applications (Meng et al., 2013), none of which were tested in our experiments. This leads onto our second aim which was to assess which method of *Bacillus* application resulted in optimum root colonisation and growth promotion effects.

Bacillus amyloliquefaciens QST 713 root colonisation data showed no obvious differences between the application methods used in the 37-day carrot experiment. This, in combination with the lack of root development data collected in this experiment meant that results were inconclusive as to which method was the most effective. None of the application methods tested reflected the root drench method used in the studies mentioned above, the closest being either the seedbed spray or the pipette treatment. However, both were applied at sowing when no roots were present, unlike in the other studies where *Bacillus* was applied after sowing.

Interestingly, in potatoes, the closer *B. amyloliquefaciens* was applied to the tuber the larger the growth promotion effects observed. As no differences were observed in carrot growth, application method appeared to have no effect. The lack of root development data also meant that assessment of growth promotion at 37 days was limited and so growth promotion effects on carrot root architecture at this stage should not be ruled out.

As plant-microbe interactions are affected by the plant species, the variety and the environmental conditions (Nowak, 1998) it may be that QST 713 does not stimulate growth of carrots under normal growth conditions. This brings me onto the final aim of the experiment, looking at the effect of low temperature on the growth promoting ability of QST 713. Following on from *in vitro* investigations on the effect of temperature on *B. amyloliquefaciens* growth we hypothesised that low temperature conditions would affect the root colonisation ability of *B. amyloliquefaciens* and any growth promotion effects which might be observed following 70 days of carrot growth. This, however, was not the case and while no growth promotion was observed under normal conditions, under low temperature conditions *B. amyloliquefaciens* treated carrots had significantly increased root length density. A trend was also observed for increased root surface area, rooting depth, shoot dry weight, leaf area and nutrient uptake of treated carrots under the lower temperature regime. The changes in carrot growth here shared many similarities to those seen in potatoes, with increases seen in root system development, nutrient uptake, shoot height and dry weight. These results suggest that *B. amyloliquefaciens* has the potential to alleviate low temperature stress in

carrots. As discussed in Chapter 5, this could be as a result of cold adaptation causing changes in the gene expression, secondary metabolite production and physiology of *B. amyloliquefaciens* QST 713 (Budde et al., 2006, Pertot et al., 2013). The change in soil temperature appeared not to reduce the ability of *B. amyloliquefaciens* QST 713 to colonise carrot roots, instead colonisation was improved. The reduced *B. amyloliquefaciens* detection down the tap root observed under the warmer temperature regime may have been due to the rapid root growth rate exceeding the *B. amyloliquefaciens* colonisation rate (Fan et al., 2012).

In this set of experiments, *B. amyloliquefaciens* QST 713 appeared to have the greatest impact upon root growth, which was seen to increase significantly in potatoes under normal growth conditions and in carrots grown under low temperature conditions, following treatment. Meng et al. (2016) made similar observations, finding the similar *B. amyloliquefaciens* strain BAC03 to have promising beneficial effects on root crops such as carrot, radish, turnip and beet.

The main limitation to this set of experiments was the natural variation between the plants. Variation needed to be accounted for by a large number of replicates, but due to the time needed to process the plants during and after harvest, the number of replicates, and therefore samples at each stage was limited. If time had not been limiting it might have been beneficial to assess the nitrogen content of the plant material as well as any differences in the nutrient content between treated and untreated soil in these experiments. For example, differences in the Olsen P of the soil might have indicated the potential for phosphate solubilisation by *B. amyloliquefaciens* QST 713 (Garcia-Lopez et al., 2018). However, as the differences in

phosphate content of both potatoes and carrots were not notably larger than differences in any other nutrients and the macronutrient content of the plant material was not significantly higher in treated plants there is no evidence to support phosphate solubilisation.

The method used to detect *B. amyloliquefaciens* QST 713 in this set of experiments also had its limitations and made observing any differences in the Serenade ASO® application methods challenging. As discussed in Chapter 3, using DNA extraction and qPCR meant the sections of roots sampled were only a very small representation of the root system, making colonisation assessment a challenge.

The detection limit of the qPCR assay used in these experiments was not hugely sensitive at 10^4 CFU g^{-1} of soil and root. The choice of extracting DNA from both soil and root as opposed to just root meant using a soil DNA extraction kit, which resulted in the presence of soil inhibitors in the DNA. Having to dilute all the DNA samples due to the presence of soil inhibitors meant diluting *B. amyloliquefaciens* DNA down, which in some cases was only present in small concentrations anyway. Alternatively, Lahlali et al. (2013) washed canola roots before performing gDNA extraction using the DNeasy Plant Mini Kit (Qiagen, Montreal, QC, Canada). This method eliminated the soil element, which may have removed any inhibitors and therefore the need to dilute DNA samples before qPCR. Mendis et al. (2018) also removed soil from maize roots, using a shaking technique, to focus purely on root colonisation. Mendis et al. (2018) go on to describe how using both soil and root could underestimate the density of *B. amyloliquefaciens* QST 713, as colonisation is higher on the root surface than in the soil. Therefore, if an increased mass of clean

root had been sampled the detection rate of *B. amyloliquefaciens* might have been higher. Using this method might have increased the *B. amyloliquefaciens* detection rates we observed in our experiments.

Mendis et al. (2018) describe a strain specific qPCR protocol recently developed for the detection and quantification of *B. amyloliquefaciens* QST 713 colonising maize roots. The protocol, which has a detection limit of 10^3 CFU g^{-1} of root, was used successfully both in controlled environment experiments and the field. The optimised assay described by Mendis et al. (2018) could therefore be used in future *B. amyloliquefaciens* QST 713 colonisation experiments.

Bacillus amyloliquefaciens QST 713 was seen here to successfully promote early potato growth, suggesting the potential for positive effects on yield. The beneficial effects of *B. amyloliquefaciens* QST 713 observed here on carrots under cold stress conditions might suggest the potential for QST 713 to alleviate abiotic stress, although further research is needed. Multiple papers (Chakraborty et al., 2015, Radhakrishnan et al., 2017) discuss the ability of *Bacillus* species to alleviate various abiotic stresses in crops, including drought, temperature stress, salinity and nutrient deficiency, and suggest the need for further research. Due to the inconclusive application method results found in this study, further work should also focus on optimising the application method used to inoculate crop roots with *B. amyloliquefaciens* QST 713.

6.1 Conclusions

In this set of experiments *B. amyloliquefaciens* QST 713 appeared to colonise the root systems of both potato and carrot plants successfully. *Bacillus amyloliquefaciens* colonisation of the carrot root system occurred even under the lower temperature conditions of 15^{o}C/10^{o}C. Growth promotion by *B. amyloliquefaciens* QST 713 was observed under optimal conditions in potatoes at 28 days but not in carrots at 21, 37 or 70 days. However, an increased carrot root system was evident in treated carrots grown under the lower temperature regime, resulting in a significantly increased root length density. The increases observed in root growth of plants treated with *B. amyloliquefaciens* QST 713 may have been responsible for the increases observed in plant nutrient uptake. Further work should now investigate the direct mechanisms involved in the enhancement of root architecture by QST 713, the optimal field application technique, and the use of *B. amyloliquefaciens* QST 713 to mitigate cold stress in crop plants.

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Chapter 8 Appendix

Table 8-1: The quantity of Bacillus amyloliquefaciens *(CFU g^{-1} of soil) found in the potato rhizosphere at various depths.*

Treatment	Depth (cm)	Plant Rep	Tm1[8]	Tm2	Cq[9]	CFU g^{-1} soil[10]
Control	2	1	80.99		39.62	ND
			79.5	82.84	38.31	ND
		2	82.42		39.34	ND
			81.72	85.92	38.55	ND
		3	76.3	82.14	41.29	ND
			76.46	82.23	41.65	ND
		4	72.61	83.12	38.05	ND
			82.27		39.75	ND
			72.26	81.07	35.61	ND
		5	81.01	83.7	35.46	ND
			75.8	82.18	37.04	ND
			83.53		35.61	ND
		6	82.78		41.22	ND
			73.85	82.86	40.12	ND
			72.39	81.71	38.22	ND
Mixed	2	1	77.24		33.77	5.79E+04
			77.31	81.8	33.3	7.97E+04
		2	78.25	81.86	32.95	9.64E+04
			81.4		36.93	ND
		3	78.34	82.36	31.94	1.66E+05
			80.24	83.49	36.08	ND
		4	78.04		32.67	1.18E+05
			77.79	81.36	35.34	ND
			78.09		34.32	3.83E+04
		5	77.71		34	5.16E+04
			78.24		34.77	3.06E+04
			77.91		33.92	5.45E+04
		6	72.89	76.59	39.13	ND
			80.79		45	ND
			83.35		36.65	ND

[8] Melting temperature
[9] Cycle threshold value
[10] ND indicates that *Bacillus subtilis* was not detected as the Cq exceeded 35 and/or the Tm was outside the range of 78 +/- 1°C.

	15		78.59	82.27	37.95	ND
		1	79.83	82.32	38.49	ND
			74.31	81.02	35.6	ND
		2	73.17	82.19	37.01	ND
			77.44	82.24	36.89	ND
		3	82.43		38.53	ND
			77.89	82.69	38.84	ND
			74.25		43.52	ND
		4	82.67		36.08	ND
			81.23		39.07	ND
			82.85		43.44	ND
		5	80.8	82.96	39.24	ND
			82.32		39.53	ND
			77.26	82.31	36.11	ND
		6	77.28	82.88	36.49	ND
	30		83.17		36.99	ND
		1	81.89		36.14	ND
			73.96	81.96	35.28	ND
		2	81.47	84.78	35.83	ND
			81.23		39.52	ND
		3	80.8		38.72	ND
			82.78	88.03	36.64	ND
			77.27	82.79	39.33	ND
		4	79.12	82.58	37.34	ND
			77.92	82.48	35.59	ND
			82.86		39.53	ND
		5	78.05	86.37	36.92	ND
			72.89	82.51	38.83	ND
			80.33	83.9	37.47	ND
		6	85.07		36.62	ND
	Root tip		82.32		37.89	ND
		1	80.86		38.95	ND
			82.7		37.96	ND
		2	80.92	86.68	38.19	ND
			77.87	81.56	34.51	3.47E+04
		3	78.78	82.34	38.48	ND
			76	83.38	38.29	ND
			73.46	82.05	37.17	ND
		4	77.9	82.93	35.53	ND

			77.97	82.04	34.83	2.41E+04
			80.91		36.5	ND
		5	78.1	81.67	35.9	ND
			83.31	88.13	37.62	ND
			73.73	82.54	37.14	ND
		6	74.1	81.93	35.82	ND
Pipette			81.32	85.15	38.08	ND
		1	81.4	83.92	39.8	ND
			76.01	78.23	37.86	ND
		2	78.09	82.22	32.35	1.19E+05
			82.58		37.49	ND
		3	78.09	81.56	37.34	ND
			78.31		30.87	4.20E+05
	2		78.18		31.15	3.47E+05
		4	78.01		31.76	2.29E+05
			72.59	83	40.29	ND
			83.25	85.81	36.76	ND
		5			37.48	ND
			82.21		37.78	ND
			83.21		36.26	ND
		6	82.04	87.18	36.6	ND
			72.39	82.26	35.06	ND
		1	76.92	81.17	37.05	ND
			78.09	83.34	34.34	ND
		2	84.1	88.62	38.22	ND
			81.03	88.6	38.97	ND
		3	81.14	88.04	35.61	ND
			83.46		35.15	ND
			78.04	83.18	35.72	ND
	15		72.59	78.84	36.97	ND
			74.35	81.59	35.71	ND
		4	73.13	83.04	45	ND
			77.24	82.08	38.83	ND
			77.66	81.44	35.13	ND
		5	79.08	82.84	37.12	ND
			82.42		39.3	ND
			77.12	81.56	36.47	ND
		6	76.89	83.63	37.28	ND

	30		77.48	82.07	35.52	ND
		1	77.81	81.83	36.51	ND
			79.53	83.38	37.64	ND
		2	76.95	88.02	37.66	ND
			80.2	82.97	40.46	ND
		3	81.34	86.27	39.93	ND
			81.1		38.66	ND
			80.32	82.72	35.95	ND
		4	77.71	82.52	36.86	ND
			81.73		37.99	ND
			83.36		37.66	ND
		5	81.65	87.99	38.61	ND
			82.39	88.27	36.83	ND
			72.95	83.63	35.56	ND
		6	79.77	82.95	36.54	ND
	Root tip		78.24		32.46	1.36E+05
		1	81.45		37.18	ND
			72.94	82.21	35.13	ND
		2	66.28	74.86	30.14	ND
			76.86	81.91	37.96	ND
		3	77.29	81.74	36.06	ND
			76.54	81.47	37.05	ND
			77.88	82.18	35.34	ND
		4	77.94		35.7	ND
			72.77	82.25	37.27	ND
			77.99	82.05	41.07	ND
		5	79.9	87.46	43.03	ND
			72.8	80.13	37.29	ND
			79.16	83.71	39.21	ND
		6	86.07		37.64	ND

Table 8-2: Bacillus amyloliquefaciens *CFU g^{-1} of soil at various depths down the carrot root system following 21 days of growth.*

Treatment	Depth	Rep	Tm1[11]	Cq[12]	CFU/g soil[13]
Control	2	1			ND
			78.15	45	ND
			70.09	45	ND
		2	69.74		ND
			73.74	43.17	ND
			83.81	45	ND
		3	82.46	45	ND
			79.04	45	ND
			75.9	44.44	ND
		4	82.57	45	ND
			76.78	45	ND
			82.16	41.08	ND
		5	81.85	41.89	ND
			82.24	44.31	ND
			81.93	42.43	ND
		6	82.37	45	ND
			79.13	38.37	ND
			73.62	41.03	ND
Treated	2	1	77.95	26.79	6.01E+06
			77.8	25.5	1.44E+07
			77.7	25.51	1.43E+07
		2	77.34	41.77	ND
			77.82	29.88	6.55E+05
			77.52	27.7	2.88E+06
		3	77.17	27.29	4.11E+06
			77.57	26.81	5.69E+06
			77.74	26.8	5.73E+06
		4	77.71	27.54	3.10E+06
			77.82	27.31	3.63E+06
			77.61	27.35	3.53E+06
		5	77.86	27.21	4.19E+06
			77.63	30.73	3.82E+05
			77.75	26.76	5.69E+06
		6	78	30.5	4.30E+05
			77.96	28.3	1.92E+06

[11] Melting temperature
[12] Cycle threshold value
[13] ND indicates that *Bacillus subtilis* was not detected as the Cq exceeded 35 and/or the Tm was outside the range of 78 +/- 1°C.

			77.64	28.29	1.93E+06
	13		79.22	41.6	ND
			72.94	45	ND
		1	78.34	45	ND
			82.43	39.54	ND
			82.17	38.6	ND
		2	77.34	33.58	4.95E+04
			78.13	36.32	ND
			70.25	45	ND
		3	80.92	37.85	ND
			74.84	45	ND
			78.95	45	ND
		4	78.11	45	ND
			72.66	45	ND
			77.73	31.65	1.85E+05
		5	78.22	45	ND
			82.37	45	ND
			82.57	45	ND
		6	82.55	45	ND
	25		82.63	41.31	ND
			77.26	37.9	ND
		1	83.05	41.53	ND
			77.4	40.44	ND
					ND
		2	82.41	42.21	ND
			67.22		ND
			77.86		ND
		3	77.72	30.16	5.66E+05
			82.49	40.71	ND
					ND
		4	78.39	44.39	ND
			77.6		ND
			77.79		ND
		5	78.1	45	ND
			73.25		ND
			78.07		ND
		6	77.72		ND

Table 8-3: Bacillus amyloliquefaciens *CFU g^{-1} of soil at various depths down the carrot rhizosphere following 37 days of growth.*

Treatment	Depth	Rep	Tm1[14]	Cq[15]	CFU g^{-1} soil[16]
Control	2	1	78.21	42.37	ND
			81.22	45	ND
			78.21	44.12	ND
		2	64.08		ND
					ND
			70.34	45	ND
		3			ND
			77.81		ND
			82.63	45	ND
		4	80.22	40.85	ND
			82.49		ND
			81.24	45	ND
		5	83.49	39.05	ND
			81.14	38.26	ND
			76.04	45	ND
		6	82.57		ND
			73.6		ND
			72.79	43.11	ND
Mixed		1	78.03	33.97	3.59E+04
			78.05	35.09	ND
			77.9	32.88	7.52E+04
		2	78.17	36.29	ND
			78.28	40.34	ND
			78.01	35.38	ND
		3	77.11	32.6	7.75E+04
			77.85	32.57	7.91E+04
			77.98	31.55	1.58E+05
		5	82.95	45	ND
			65.45		ND
			77.73		ND
		6	77.45	34.61	2.05E+04
			77.31	34.19	ND
			77.46	32.29	9.94E+04
	10	1	74.98	36.11	ND
			78.37	37.38	ND

[14] Melting temperature
[15] Cycle threshold value
[16] ND indicates that *Bacillus subtilis* was not detected as the Cq exceeded 35 and/or the Tm was outside the range of 78 +/- 1°C.

			78.36	34.85	1.84E+04
			78.15	38.58	ND
			80.73	45	ND
		2	78.51	44.3	ND
			81.16	45	ND
			81	42.96	ND
		3	80.71	40.22	ND
			74.01	40.27	ND
			78.49	38.16	ND
		5	80.23	40.37	ND
			73.44	45	ND
			73.47	41.66	ND
		6	74.4	42.08	ND
	20		73.03		ND
			73.77		ND
		1	79.4	45	ND
			81.88	45	ND
					ND
		2	80.61	39.25	ND
			77.99	37.44	ND
			79.6	45	ND
		3	80.55	41.11	ND
			66.96		ND
			66.71		ND
			77.9	37.52	ND
		5	77.69	36.34	ND
			77.59		ND
			77.62		ND
		6	77.63		ND
	Bottom		77.95	37.54	ND
			77.78	38.49	ND
		1	77.89	35.29	ND
			78.14	38	ND
			78.05	36	ND
		2	72.85	38.74	ND
			78.03	37.08	ND
			80.12	43.8	ND
		3	80.05	44.59	ND
			78.21	42.19	ND
			78.08	40.15	ND
		5	77.78	34.96	1.77E+04
			77.83	35.79	ND
			77.71	34.41	2.31E+04
		6	77.74	33.76	3.60E+04
Pipette	2	1	78.45	45	ND

			78.27	44.27	ND
			78	29.64	7.63E+05
			74.58		ND
			78.04		ND
		2	77.98	33.79	3.28E+04
			78.05	30.53	3.81E+05
			78.07	30.63	3.56E+05
		3	78	29.68	6.79E+05
			66.36		ND
			66.47		ND
		5	74.16		ND
					ND
			77.7	31.62	1.92E+05
		6	78.08	44.57	ND
			77.75		ND
			83.54	43.28	ND
		1	78.3	45	ND
			81.64	40.03	ND
			74.08	37.96	ND
		2	72.35	39.17	ND
			78.17	38.34	ND
	10		80.83	45	ND
		3	81.25	38.27	ND
			78.61	40.79	ND
			83.32	42.44	ND
		5	80.08	36.63	ND
			81.33	35.1	ND
			81.42	37.75	ND
		6	82.9	36.18	ND
			80.84	37.16	ND
			81.11	39.92	ND
		1	81.93	37.59	ND
			82.21	39.66	ND
			82.57	38.21	ND
		2	77.67	35.06	ND
			74.37	40.06	ND
	20		80.77	44.3	ND
		3	81.24	41.58	ND
			69.1		ND
			73.37		ND
		5	77.95	36.16	ND
			78.29	42.28	ND
			73.7	42.4	ND
		6	79.96	40.25	ND
	Bottom	1	77.82	33.27	6.11E+04

			77.87	31.89	1.56E+05
			77.88	32.9	7.86E+04
			77.15	34.66	2.11E+04
			77.78	35.54	ND
		2	78.01	34.13	3.03E+04
			77.84	36.01	ND
			79.91	41.21	ND
		3	77.9	36.55	ND
			77.73	45	ND
			81.28	37.06	ND
		5	79.45	38.51	ND
			71.72		ND
			72.93	39.76	ND
		6	80.56	41.82	ND
Spray	2		78.09	34.39	2.82E+04
			77.94	32.24	1.22E+05
		1	77.98	31.66	1.80E+05
			78.18		ND
			78.18		ND
		2	69.6	45	ND
			78.18		ND
			78.25	35.12	ND
		3	78.16	35.48	ND
			78.25		ND
			67.75		ND
		4	80.54	42.07	ND
			77.89	30.72	3.70E+05
			77.67	31.03	3.00E+05
		5	72.64	40.18	ND
			77.92	32.95	7.46E+04
			76.95		ND
		6	77.9	34.74	2.21E+04
	10		77.59	40.31	ND
			80.53	45	ND
		1	78.83	45	ND
			81.02	45	ND
			82.83	43.22	ND
		2	82.77	38.08	ND
			78.13	39.92	ND
			82.05	37.98	ND
		3	82.75	45	ND
			81.24	38.02	ND
			72.17	43.44	ND
		4	77.97	35.81	ND
		5	79.32	38.96	ND

			80.23	38.69	ND
			80.72	40.87	ND
			81.15	45	ND
			80.74	39.39	ND
		6	82.76	36.79	ND
			66.29		ND
			80.1		ND
		1	81.5	38.53	ND
			79.66	45	ND
			80.02	39.22	ND
		2	83.31	45	ND
			72.17	38.59	ND
			81.54	40.32	ND
		3	80.72	45	ND
	20		77.41	33.36	ND
			77.68		ND
		4	77.74	35.18	ND
			77.46	33.79	4.01E+04
			77.61	33.79	4.01E+04
		5	72.65	39.01	ND
			77.74	34.86	1.53E+04
			77.83		ND
		6	77.61	39.2	ND
			77.24	36.23	ND
			77.87	35.55	ND
		1	77.76	34.12	2.81E+04
			82.77	43.19	ND
			78.76	42.45	ND
		2	72.32	45	ND
			82.04	38.76	ND
			78	38.08	ND
		3	78.29	43.28	ND
	Bottom		79	45	ND
			80.71	44.29	ND
		4	82.04	37.63	ND
			77.5	42.36	ND
			82.07	37.86	ND
		5	77.97	35.49	ND
			78.1	37.04	ND
			78.26	35.06	ND
		6	77.93	36.73	ND
1TL	2		77.94	29.3	9.03E+05
			77.92	29.63	7.22E+05
		1	77.98	28.74	1.32E+06

			66.27		ND
			67.36		ND
		2	78.18	35.44	ND
			78.3	33.19	6.90E+04
			78.17	33.26	6.58E+04
		3	78.21	32.46	1.13E+05
			78.09	36.82	ND
			78.14	35.04	ND
		4	78.21	35.02	ND
			77.13	36.28	ND
			77.5	33.69	3.67E+04
		5	77.41	34.42	ND
			77.73	32.23	1.34E+05
			77.64	30.92	3.27E+05
		6	77.49	31.34	2.46E+05
					ND
					ND
		1			ND
			80.29	41.9	ND
			77.46	35.25	ND
		2	79.56	40.74	ND
			78.01		ND
			69.49	45	ND
		3	80.61	45	ND
	10		77.9		ND
			81.11	45	ND
		4	80.79	45	ND
			81.18	39.66	ND
			78.31	37.21	ND
		5	79.35	35.81	ND
			80.94	44.21	ND
			80.79	39.69	ND
		6	76.67	37.97	ND
					ND
			72.44	45	ND
		1	78.16	41.23	ND
			77.28	35.05	ND
			77.99	41.09	ND
	20	2	77.21	35.75	ND
			78.05	36.99	ND
			78.1	36.28	ND
		3	81.42	35.95	ND
		4	81.87	43.07	ND

			82.59	45	ND
			82.69	45	ND
			80.51	38.31	ND
			81.4	40.26	ND
		5	80.46	45	ND
			73.95	37.11	ND
			80.72	38.89	ND
		6	82.7	45	ND
	Bottom		78.12	36.68	ND
			77.71	33.94	4.01E+04
		1	77.65	34.85	2.16E+04
			77.8	36.78	ND
			77.84	33.87	3.49E+04
		2	81.24	36.42	ND
			78.25	40.99	ND
			77.85	35.89	ND
		3	77.61	34.92	ND
					ND
			65.53		ND
		4	81.7	45	ND
			77.74	34.89	1.42E+04
			77.48	34.62	1.70E+04
		5	77.79	33.67	3.25E+04
			68.9		ND
			80.5	41.84	ND
		6	77.96	37.68	ND

Table 8-4: Bacillus amyloliquefaciens *CFU g^{-1} of soil at various depths down the carrot root system following 70 days of growth under high and low temperature regimes.*

Temperature	Treatment	Depth	Rep	Tm1[17]	Cq[18]	CFU g^{-1} soil[19]
High	Control	0-15		75.08	40.33	ND
						ND
			1	78.94	41.75	ND
				78.06	38.65	ND
				84.05	40.78	ND
			2	77.94	37.08	ND
				74.23	37.15	ND
				71.65	42.37	ND
			3	79.29	38.39	ND
				81.78	35.96	ND
				77.85	37.34	ND
			4	82	35.57	ND
				77.9	37.17	ND
				78.77	37.74	ND
			5	72.53	40.72	ND
				78.43	43.33	ND
				72.44	40.63	ND
			6	77.54	37.53	ND
				78.05	38.96	ND
				72.46	39.48	ND
			7	78.67	41.45	ND
				80.43	45	ND
				79.51	45	ND
			8	80.38	39.96	ND
High	Treated	0-15		77.57	31.94	1.48E+05
				76.76	31.79	ND
			2	77.69	32.24	1.20E+05
				78.68	39.98	ND
				78.4	40.3	ND
			3	77.96	31.67	1.98E+05
				76.82	45	ND
				78.31	32.78	9.20E+04
			4	77.22	34.83	2.28E+04
				78.27	29.58	9.18E+05
			5	78.33	29.51	9.63E+05

[17] Melting temperature

[18] Cycle threshold value

[19] ND indicates that *Bacillus subtilis* was not detected as the Cq exceeded 35 and/or the Tm was outside the range of 78 +/- 1°C.

				78.22	29.97	7.04E+05
				78.19	29.71	6.69E+05
				78.29	29.2	9.47E+05
			6	78.08	29.2	9.47E+05
				78	30.93	3.46E+05
				77.92	31.18	2.92E+05
			7	77.94	30.56	4.45E+05
				78.28	29.36	8.94E+05
				77.96	29.05	1.10E+06
			8	68.57	40.9	ND
High	Treated	30-45		77.47	31.83	1.80E+05
				81.1	41.87	ND
			1	77.88	29.88	6.77E+05
				82.68	42.98	ND
				82.34	39.87	ND
			2	82.56	43.06	ND
					38.57	ND
				75.88	41.71	ND
			3	79.33	42.78	ND
					37.58	ND
				82.69	37.75	ND
			4	77.36	38.7	ND
				82.89	38.26	ND
				74.45	35.81	ND
			5	78.3	35.78	ND
				79.56	38.18	ND
				79.06	41.58	ND
			6	81.29	39.28	ND
				78.15	35.02	ND
				77.92	35.25	ND
			7	81.86	40.46	ND
				79.47	45	ND
				77.53	43.08	ND
			8	82.25	39.35	ND
High	Treated	75-90		81.03	38.72	ND
				78.17	37.79	ND
			1	80.82	39.17	ND
				72.75	43.24	ND
				82.15	42.86	ND
			2	76.99	36.82	ND
					36.7	ND
				73.27	37.53	ND
			3	82.28	37.73	ND
				73.29	40.59	ND
			4	72.77	37.3	ND

				78.12	36.72	ND
				75.67	36.73	ND
				78.4	35.28	ND
			5	83.3	36.61	ND
				80.02	45	ND
				80.45	36.06	ND
			6	83.05	39.92	ND
				84.18	35.7	ND
				84.77	35.4	ND
			7	84.53	35.39	ND
				73.04	43.34	ND
				77.95	36.84	ND
			8	79.26	42.68	ND
High	Treated	Bottom		79.85	39.75	ND
				82	40.65	ND
			1	72.78	41.51	ND
				78.85	40.09	ND
				81.67	40.5	ND
			2	81.33	37.73	ND
				75.85	44.18	ND
				86.93	39.99	ND
			3	83.66	39.05	ND
				68.15	40.58	ND
				77.85	39.14	ND
			4	78.09	36.37	ND
				72.99	35.49	ND
				77.22	37.6	ND
			5	72.53	35.15	ND
				82.24	38.72	ND
				72.47	36.05	ND
			6	78.05	34.91	ND
				84.81	39.23	ND
				80.41	34.98	ND
			7	81.38	38.06	ND
				83.41	41.05	ND
				81.87	39.05	ND
			8	72.35	40.3	ND
Low	Control	0-15		78.71	45	ND
				82.19	45	ND
			1	72.72	42.7	ND
				78.25	35.18	ND
				82.39	37.87	ND
			3	76.31	36.01	ND
				77.88	37.44	ND
			5	82.41	39.46	ND

				73.7	41.32	ND
					45	ND
				73.49	38.78	ND
			6	82.37	38.21	ND
				78.52	45	ND
				72.55	38.67	ND
			8	79.39	39.69	ND
Low	Treated	0-15		78	29.98	6.94E+05
				77.9	30.18	6.06E+05
			1	77.83	30.18	6.06E+05
						ND
				78.31	38.01	ND
			2	77.96	31.05	3.12E+05
				78.26	31.24	2.95E+05
				78.25	29.97	6.99E+05
			4	78.35	29.97	6.99E+05
				78.32	31.6	2.31E+05
				78.25	32.74	1.06E+05
			5	78.19	32.3	1.43E+05
				78.6	45	ND
				72.53	41.46	ND
			6	78.19	31.72	1.72E+05
				78.14	31.77	1.73E+05
				78.32	31.83	1.66E+05
			7	78.27	31.04	2.84E+05
				78.16	29.07	1.46E+06
				78.2	29.33	1.22E+06
			8	78.19	29.03	1.50E+06
Low	Treated	30-45		80.41	41.9	ND
				78.29	45	ND
			1	77.37	40.51	ND
				77.95	34.5	3.13E+04
				77.88	35.3	ND
			2	77.69	35.67	ND
				78.05	35.11	ND
				80.82	40.68	ND
			4	79.7	39.78	ND
				83.88	38.94	ND
				79.65	39.32	ND
			5	81.45	41.91	ND
				80.78	36.57	ND
				72.48	39.84	ND
			6	79.41	43.79	ND
				79.99	42.87	ND
			7	73.87	40.29	ND

				79.56	41.71	ND
				73.86	41.34	ND
				83.05	45	ND
			8	81.78	42.15	ND
				86.44	35.86	ND
				79.68	41.26	ND
			1	82.37	38.47	ND
				78.6	39.1	ND
				80.99	42.78	ND
			2	80.53	38.22	ND
				74.3	36.92	ND
				74.19	39.78	ND
			4	77.89	44.45	ND
				79.92	34.15	ND
Low	Treated	75-90		79.43	34.07	ND
			5	81.44	36.91	ND
				78.29	37.26	ND
				79.8	39.01	ND
			6	77.93	34.45	2.84E+04
				75.95	42.92	ND
				81.66	42.63	ND
			7	78.17	36.09	ND
				69.62	45	ND
				77.87	43.28	ND
			8	82.62	42.85	ND
				76.84	34.85	2.07E+04
				77.68	35.78	ND
			1	79.41	40.48	ND
				81.87	41.76	ND
				82.47	39.88	ND
			2	81.18	37.99	ND
				73.3	37.77	ND
				78.91	35.72	ND
			4	83.1	45	ND
				81.94	38.85	ND
Low	Treated	Bottom		72.83	36.88	ND
			5	78.37	35.31	ND
				72.91	36.25	ND
				78.25	33.74	5.89E+04
			6	78	32.64	1.24E+05
				82.17	40.84	ND
				82.05	40.31	ND
			7	81.71	37.47	ND
				82.78	37.28	ND
			8	86.47	37.6	ND

				80.3	37.73	ND