Effect of *Bacillus coagulans* DUS-M6 strain and its pyoverdine-like siderophore on *Brassica napus* (canola)

by

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Master of Science (Microbial Biotechnology)

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Master of Science (Research)

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Abstract

Background

Plant growth promoting bacteria (PGPB) are soil dwelling microbes that enhance plant growth via a number of different direct and indirect processes. These beneficial microbes are gaining increasing interest for development and use on plant crops as they can reduce inputs requirements and their associated input costs and increase overall crop yield mass and yield quality. Currently, there are no commercially available *Bacillus* based inoculants for use with canola that reliably enhance plant growth and yield or are particularly tailored for Australian cropping conditions. This is largely due to incomplete characterizations, undetermined modes of action and incompatibility across cropped plants and cropping systems.
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1.0 Introduction

Due to increase in growing population and to feed it the increase for food has made essential for extensively use of certain chemical fertilizers in the agricultural lands which led to polluting the underground water bodies through leaching and erosion. Public concerns are arising due to overuse of chemical fertilizers. If crops containing residues of the pesticides and they are consumed, then it may be hazardous to the humans. So, to combat such situations efforts are being made to find an alternative solution to the chemical fertilizers in the form of beneficial soil bacteria which are believed to increase the growth and productivity of the plants on when they are applied in the rhizosphere of the plant. These beneficial rhizobacteria which enhances the growth and productivity of the plants are called as plant growth promoting rhizobacteria (PGPR).

When these are applied on the roots or seeds of the plants they colonize the whole root system and further utilize the root exudates which are in the form of amino acids, sugars as the source of their nutrients and initiates growth promoting activities which directly enhances the growth and productivity of plant (Kloepper and Schroth, 1978).

It has been shown that when plants are inoculated with PGPR they show an increase in the growth by 500% (Kloepper et. al. 1980) and productivity by 57% (Asghar 2004). PGPR increases plant growth by providing the essential nutrients and hormones directly to the rhizosphere or by reducing the activity of plant pathogens indirectly (Ahemad & Kibret 2014). These bacteria which are responsible for reducing the activity of plant pathogens are known as biocontrol agents (BCA). These biocontrol bacteria which promotes the plant growth are called as PGPR. (Ahemad & Kibret 2014). Not all BCA is known to enhance the plant growth, therefore the term PGPR is not applied for all BCA. Many of the PGPR have been identified and further developed into commercial products (Junaid 2013).

Recent research is going on identifying the new strains or improving the existing ones which has already been found to promote plant growth.

The most commonly known PGPR are Agrobacterium, Azospirillum, Azotobacter, Bacillus, Burkholderia, Chromobacterium, Caulobacter, Erwinia, Micrococcus, Pseudomonas and Serratia (Bhattacharyya & Jha 2012). Currently scientists are focusing on endospore forming bacteria gram positive belonging to genera Bacillus, Paenibacillus and Lysinibacillus. This was formally classified as Bacillus but was separated into different genera (Xu and Cote, 2003). Because of endospores which are stress tolerant provides
bioformulations and has long shelf life and high survival rates under adverse conditions (Thomas, 2012; Mandic-Mulec and Prosser, 2011: Adesemoye et al., 2017). Because of these characteristics they make a good option as commercially available PGPR inoculants.

1.1 Stealing iron from environment and providing them to the plants

Iron is an important element which is required for the growth of almost all the living creatures. It is essential for cellular processes such as the electron transport chain, synthesis of nucleic acids and the catalytic reactions of many enzymatic activities (Aguado-Santacruz GAA 2012).

It is present as the fourth most abundant element in the earth’s crust after oxygen, silicon and aluminium. Iron can exist in two states when present in the aqueous phase: Fe$^{2+}$ and Fe$^{3+}$ but often at biological pH and under certain aerobic conditions it is oxidised to insoluble polymeric forms of oxyhydroxides which results in limited bioavailability and therefore are not utilized by plants and microbes (Desai and Archana 2011; Zuo and Zhang 2011). Due to this, it has been estimated that almost one-third of the soil on earth has iron deficiency (Yi et al., 1994). The concentration of available ferric iron in the environment is around $10^{-18}$ M and the concentration required by the plants to maintain their internal iron requirement is about $10^{-6}$M (Raymond et al., 2003). Therefore, the low limitation of iron can lead to some unavoidable threats for the microorganisms, they have developed some specific mechanisms to overcome this iron limitation such as production of siderophores. These are low molecular weight (200-2000Da) metal chelating compounds which are secreted in the form of secondary metabolites by plants and microorganisms under low iron concentrations (BERNHARD SCHWYNAND 1987). These molecules are small peptides with side chains and functional groups which can scavenge iron and further transport through cell membrane (Raymond et al., 2015). There are two known strategies known by plants to obtain iron: (i) acidification of the rhizosphere atmosphere and then reduction of Fe$^{3+}$ ions which is done by membrane bound Fe$^{3+}$ reductase and uptake of Fe$^{2+}$ by the roots. (ii) secretion of Phyto siderophores which are able to solubilise and bind iron which is further passed to the root cells through membrane bound proteins (Altomare and Tringovska 2011; Guerinot 2010).
These strategies involved sufficient to meet the requirement of the plants specially growing in alkaline and calcareous soil. Therefore, plants need to be given essential concentration of iron in the iron limiting conditions (Zuo & Zhang 2011). The strategies involved to deliver iron to plants are through crop and soil management in which iron is delivered through root of foliar in inorganic form of iron (FeSO₄) or synthetic and non-synthetic iron chelates (Fernàndez, Ebert & Winkelmann 2005) whereas in soil management fertilization is done with inorganic salts such as NO₃⁻ or NH₄⁺ which brings changes in the pH which further improves the solubility of soil and increase uptake (Zuo and Zhang 2011). It also involves delivering chelated forms of iron to the soil and the use of synthetic chelates such as Fe-EDTA or Fe-EDDHA has proved to be a cheaper and effective form (Shenker and Chen 2005), however, its overuse can have many negative impacts on the environment (Adesemoye, Torbert & Kloepper 2009)

1.2 Microbial iron solubilisation

Another way of fertilising soil which is currently becoming important for farmers is the application of plant growth promoting rhizobacteria (PGPR) which is a consortium of many beneficial microorganisms. These are believed to release metal-chelating compounds with molecular mass below (200-2000Da) known as siderophores (Schwyn and Neilands, 1987) in iron limiting conditions. Microbially produced siderophores bind iron via presence of functional groups such as catechols, carboxylates or hydroxymates (Raymond et al., 1984). There are also certain microbial siderophores which have a combination of different functional groups (Cornelis 2010). The fungal siderophore is known to have hydroxamates which belongs to ferrichrome family and which is divided in five groups depending on the side chain of hydroxamate (Renshaw et al. 2002; Weinberg 2004). Gram positive and gram-negative bacteria exhibit variety of siderophore Fe transport mechanisms.

The exact mechanism through which microorganisms provide iron to the plants is still largely unknown. The possible mechanism through which microbial siderophores provide Fe to the plants are: (i) the redox potential of the microbial siderophores can be further reduced to provide Fe²⁺ to plants transport system. Under this mechanism the Fe³⁺ from microbes are transported to the plant roots apoplast where siderophores are reduced (Harald Kosegarten 1999; Mengel 1994). Fe²⁺ is stored in the apoplast which leads to the high concentration of
Fe in the roots (Harald Kosegarten 1999) (ii) Microbial siderophores are able to chelate iron from its surrounding soil and this leads to ligand exchange with the phytosiderophores (Jaber Masalha & Mengel 2000). This depends on several factors such as concentration and stability of microbial siderophores and phytosiderophores and also the redox and pH of the plant roots (Crowley, 2006).

*Bacillus subtilis* is considered as a prototypical for studying the iron uptake in the gram-positive organisms. It is considered as the model organism which is known to produce siderophore types such as hydroxamate and catecholate which includes petrobactin, corynebactin, bacillibactin, schizokinen or itoic acid. Bacillibactin is a catecholate form of siderophore which a cyclic trimeric ester is and made up of three units of 2,3-dihydroxybenzoate-glycine-threonine which has iron-chelating capabilities and is attached through lactone linkages in a cyclic manner (Ito 1993). The operon of bacillibactin consists of five genes which have their own functions. The molecular analysis of the iron uptake in the *Bacillus subtilis* was first demonstrated by Schneider and Hantke in 1993. The uptake of Fe$^{3+}$ through bacillibactin in *Bacillus subtilis* requires a number of membrane-bound proteins which includes ATPase, substrate-binding proteins, permases and transporters (Khan, Doshi & Thakur 2016). FeuABC is a membrane bound transporter of bacillibactin of *B. subtilis* whereas, FepDG heterodimeric membrane-bound inner membrane permease which helps in transportation of bacillibactin. YusV is an ATP-binding protein which provides energy for the transportation of ferri-bacillibactin. YuiI is responsible for hydrolysing bacillibactin and also ferri-bacillibactin through which iron becomes freely available for carrying out the cellular processes and further for recycling of bacillibactin. The complete process of iron uptake is regulated by Fur homologue which attaches to the Fur box. There have been recent studies which supports Mta which is considered as another bacillibactin pathway regulator known as Mer-R type transcriptional regulator which activates release of bacillibactin (Miethke, Schmidt & Marahi et al 2008). The mechanism of binding between Mta and Fur homologue with metal ions and Fur box is not completely explained therefore, it requires further studies.
1.3 Antimicrobial property of siderophores

Siderophores are considered to be environmental friendly which can be used as alternative towards pesticides (Schenk, Carvalhais & Kazan 2012). Therefore, microbial siderophores have been considered as biocontrol agents against soil borne phytopathogens. These are believed to be useful for disease management and plant growth promotion. It has been shown that different species of *Pseudomonas* produce siderophores such as pyoverdines which are responsible for plant growth promotions and are also believed to show antimicrobial activities against different pathogens (Kloepper JW et al. 1980); Gamalero and Glick, 2011). *Azadirachta indica* are also believed in production of ferrioxamines which can contribute in the uptake of Fe for the plants which can lead to root and shoot growth promotion (Siebner-Freibach H 2003; Verma, Singh & Prakash 2011). Mycorrhizal fungi as biofertilizer can be used in plant growth promotions. For example, Mycorrhizal sorghum can take up higher concentrations of Fe than non-mycorrhizal plants (Caris C 1998). Fungal siderophores produced by ectomycorrhizal fungi are believed to contribute in plant nutrition (van Schöll et al. 2007).

The significant role in the biological control by siderophores has been reported by Kloepper in 1980. The underlining mechanism involves siderophores as competitors for the take up of Fe which reduces the availability of Fe for phytopathogens (Beneduzi et al., 2012). There are
many studies which are been presented for biological control activity of siderophores against many phytopathogens. Pseudomonads produce pyoverdine siderophores which have been recorded to control the wilt disease of potatoes by *Fusarium oxysporum* (Schippers B 1987) as well as *Gaeumannomyces graminis* which brings growth deficiency in barley and wheat (Voisard C 1989). In addition, it is involved in biocontrol of plant pathogens of peanuts and maize (Pal et al. 2001). Siderophores of *Bacillus subtilis* are involved in the biocontrol of *Fusarium oxysporum* which causes wilt diseases of pepper (Yu et al. 2011). Siderophore of *Azadirachta indica* has been investigated to have high potential to chelate Fe$^{3+}$ present in soil which causes hindrance in the growth of many fungal pathogens (Verma et. al., 2011)

### 1.4 Biochemistry of siderophore production

The bacterial species which are responsible for the production of siderophores has Fur homologue which regulates the biosynthesis of siderophore genes (Fig. 2). Under high iron concentrations the Fe$^{2+}$ which is a cofactor, binds with Fur proteins and further this complex bind with DNA present in the promoter of the genes which have a Fur box. Fur bound with the Fur box promotes repressions in the expression of the genes which includes siderophore biosynthetic and also ferric-siderophore uptake genes. But under low iron concentration in the soil environment the Fur protein will not bind with Fe$^{2+}$. The iron free form of the Fur, Apo-Fur is unable to bind DNA and therefore gene repression is reversed. Additional regulation methods of siderophore systems include expression of sigma factors or certain activators for additional regulation. Siderophores such as pyoverdine, pyochelin present in the *P. aeruginosa* use a combination of sigma factors and activators which regulates the expression of both the systems involved. Fur repression occurs only for certain set of siderophore genes which also includes pvdS pvhR but not for synthesis of siderophores or uptake of genes (Ochsner 1995; Visca 1994). After formation of PvdS and PchR they are responsible for upregulation of siderophore genes. During iron starvation, the repression of Fur leads to the transcription of *pvdS* and *pchR* genes. The freshly translated PvdS is in an inactive form and requires binding of Fe-pyoverdine at its outer membrane receptors for its activation (Lamont et al. 2002). PvdS is an alternative sigma factor that brings RNA polymerase for pyoverdine synthesis and uptake of genes (Braun, Mahren & Ogierman 2003; Cunliffe 1995; Wilson, McMorran & Lamont 2001) and PchR behaves as coactivator which mediates the pathway of pyochelin regulation. It is activated by Fe-pyochelin in the cell and
which is known as a member of AraC regulators results in activation of synthesis of pyochelin and uptake of certain genes and has is able to repress its own expression. Once the transcription of siderophore synthesis and uptake is completed the siderophores are assembled via multiple steps.

Fig 2. Mechanism of siderophore regulation which shows Fur repression which occurs in *P. aeruginosa*. [A] Under high iron concentration, Fur binds with the $\text{Fe}^{2+}$ which dimerises and binds with the promoter of the genes having Fur box which results in repression of the transcription. [B] During low iron concentration, Fur is not able to bind with $\text{Fe}^{2+}$ which further
does not bind with the DNA which leads to transcription of the pvdS and pvdR siderophore genes. [C] After activation via pyoverdin outer membrane receptor, pvdS brings RNA polymerase to the promoter of pyoverdine genes for transcription. [D] pcdR behaves as a coactivator for pyochelin genes after dimerizing and binding to Fe-pyochelin. (Khan, Doshi & Thakur 2016)

1.5 Siderophore assembly

Siderophores are synthesised through two pathways: (i) the amino acids are assembled into peptides bound by peptide bonds and that this doesn’t requires an RNA template. Large, multi-domain and multi-functional proteins are involved for the assembly in this pathway and are known as non-ribosomal peptide synthases (NRPSs) (Crosa & Walsh 2002). The second pathway which is the NRPS-independent pathway, has diamine or amino alcohol building blocks linked with dicarboxylic acid that are bound through amide or ester bonds (Challis 2005). It is used for the assembly of carboxylate or hydroxymate siderophores which include alcaligin, stephlobactin, aerobactin and petrobactin (Challis 2005; Miethke & Marahiel 2007).

The NRPS-dependent pathway is responsible for producing enterobactin, pyochelin, mycobactin or pyoverdine (Ackerley, Caradoc-Davies & Lamont 2003; Mossialos, N. & P. 2002; Quadri 2000). The pathway is not determined by the RNA template but by the domain for the assembly (Crosa & Walsh 2002). There are three functional domains which are required for incorporating one amino acid into the peptide chain and thus form a single module which consists of condensation, adenylation and also peptidyl-carrier domain (Crosa & Walsh 2002; Fischbach 2006). The 50kDa condensation domain is responsible for the catalyses of peptide bonds which leads to chain elongation (Crosa & Walsh 2002).

VibH was the first condensation domain that was determined, and which carries vibriobactin biosynthesis in Vibrio cholera (Keating et al. 2002). The C functional domain contains two structural domains which consists of $\alpha$-$\beta$-$\alpha$ in the form of a sandwich. The modification of the functional domain is the cyclization domain which catalyses the formation of peptide bond which is similar to the condensation domains but also cyclizes serine, threonine or cysteine which forms a five membered ring. This ring structure is further dehydrated to form a thiazoline or oxazoline ring. The other functional domain is the adenylation domain (50kDa)
which conducts selection and activation of the amino or aryl acid in an ATP-dependent manner and which further binds with the peptide (Crosa & Walsh 2002). Following activation, it forms an aminoacyl-AMP which is transferred to the peptidyl carrier domain. PheA activates phenylamine to be incorporated in the gramicidin antibiotic and DhbE, which activates 2,3-dihydroxybenzoate (DHB) also aryl acid present in bacillibactin found in Bacillus subtilis have been also determined (Conti 1997; May et al. 2002). The peptidyl-carrier domain is the last functional domain present in the NRPS module or aryl-carrier domain is then for aryl acids (Crosa & Walsh 2002). It is a small domain of 8-10kDa which contains a conserved portion of serine residue that is modified transcriptionally by adding phosphopantethionyl tail (pPant). This tail binds covalently to the activated aminoacyl-AMP which results in elongation of the chain. The tail binds with the peptidyl carrier domain through phosphopantetheinyl transferase enzyme (PPTase). There are different PPTase for every pathway which are directly involved in bringing out the primary and secondary metabolism. It has been determined that the PPTase involved in Bacillus subtilis for siderophore production is Sfp and YbtD for Yersinia pestis (Bobrov, Geoffroy & Perry 2002; Quadri & T. 1998). PcpS present in the P. aeruginosa carries out the primary and secondary metabolism. (Barekzi et al. 2004).

There are several other domains for modification of the elongating peptide in addition to the functional domains required in NRPS module, such as methyl transferase, epimerase, thioesterase domains (Crosa & Walsh 2002). The Epimerization domain results in conversion of L-amino acids into D-amino acids whereas, the methyltransferase domain uses the adenosyl methionine as the methyl donor which leads to methylation of amino acids. The thioesterase domain is responsible for termination of the elongating chain and release of the complete formed siderophore. P. aeruginosa and Yersinia have another set of additional accessory proteins which uses NADPH-dependent reductase for modification of siderophores in which the second ring of thiazoline ring of pyochelin and yersiniabactin are converted into thiazolidine ring (Geoffroy 2000; Reimmann et al. 2001). The biosynthesis of pyoverdine and pyochelin present in P. aeruginosa is NRPS-dependent and this shows how two different siderophores are assembled. Although the synthesis of yersinibactin which is similar to synthesis of pyochelin, it uses NPRS-dependent and independent pathways. The biosynthesis of these siderophores illustrates a good example of siderophore synthesis which encompass mostly other siderophore formation.
1.6 The biosynthetic pathway of Bacillibactin

Bacillibactin is produced by *Bacillus subtilis* under iron limiting conditions which is secreted to the external environment where it binds with the Fe$^{3+}$ with high specificity and affinity. The Fe$^{3+}$-siderophore complex known as ferri-siderophore complex is taken up into the cell by certain specific transporters (Stintzi et al. 2000).

Fig 3: Biosynthetic pathway of Bacillibactin which is produced by *Bacillus subtilis* under iron limiting conditions where it binds with the Fe$^{3+}$ with high specificity and affinity (Khan, Doshi & Thakur 2016)
1.7 The ability of siderophores to chelate metals other than iron

Stable complexes are known to form between siderophores other than iron (Hernlem 1996). It has been reported that the constant formation for hydroxamate siderophore desferrioxamine B with $\text{Al}^{3+}, \text{Ga}^{3+}$ and $\text{In}^{3+}$ are between $10^{20}$-$10^{28} \text{ M}^{-1}$, and $10^{30} \text{ M}^{-1}$ for $\text{Fe}^{3+}$ (Evers 1989). It has been revealed that the siderophores of Pseudomonas aeruginosa i.e. pyoverdine and pyochelin are able to chelate 16 different metals ($\text{Mn}^{2+}, \text{Ag}^{+}, \text{Al}^{3+}, \text{Co}^{2+}, \text{Cu}^{2+}, \text{Ga}^{3+}, \text{Hg}^{2+}, \text{Pb}^{2+}, \text{Sn}^{2+}, \text{Zn}^{2+}, \text{Tb}^{3+}, \text{Ni}^{2+}, \text{Eu}^{3+}, \text{Ti}^{4+}, \text{Cr}^{2+}, \text{Cd}^{2+}$) (Braud et al. 2009a; Braud et al. 2009b). Pyoverdine forms constants with $\text{Zn}^{2+}$, $\text{Cu}^{2+}$, and $\text{Mn}^{2+}$ between $10^{17}$ and $10^{22} \text{ M}^{-1}$ and $10^{32}$ with $\text{Fe}^{3+}$ (Chen 1994).

1.8 Siderophore production is also regulated by metals other than iron

The biosynthesis of siderophores by bacteria is regulated by the amount of iron in the environment and also by the quantity of iron acquired by the bacteria. It has been reported that the production of pyoverdine and pyochelin by P. aeruginosa is repressed in presence of iron by the Fur proteins (Lee & Helmann 2007). Other metals are also known to stimulate or inhibit siderophore production in various bacteria, even in presence of high concentration of iron. It has been observed that the presence of molybdenum stimulates the production of azotochelin which is a catecholate siderophore and is able to chelate this metal. At $100 \mu \text{M}$ the synthesis of azotochelin is activated whereas at higher concentration of metals the stimulation of siderophores is repressed (Duhme 1998). At higher concentration of aluminium in succinate medium (which is iron-limited) the production of hydroxamate that are schizokinen and N-deoxyschizokinen is increased but not in iron-repleted cultures of Bacillus megaterium (Hu 1996).

In succinate medium the production of pyoverdine is stimulated by $10 \mu \text{M} \text{Al}^{3+}$ addition to $\text{Mn}^{2+}$, $\text{Cu}^{3+}, \text{Ga}^{3+}$ and $\text{Ni}^{2+}$ (Braud et al. 2009b). During iron limited conditions an exposure to $10 \text{mM} \text{Cu}^{2+}$ brings upregulations in the genes that are involved in synthesis of pyoverdine and downregulates genes involved in production of pyochelin (Teitzel et al. 2006). In presence of $100 \mu \text{M}$ of iron pyoverdine production is increased to 10 and $100 \mu \text{M} \text{Cu}^{2+}$ and $\text{Ni}^{2+}$ and lesser by $100 \mu \text{M} \text{Cr}^{2+}$ (Braud et al. 2010) and during iron limited conditions no pyoverdine production was activated but $10 \mu \text{M} \text{Co}^{2+}$ and $100 \mu \text{M} \text{Mo}^{6+}, \text{Cu}^{2+}, \text{Ni}^{2+}$ showed repression (Visca 1992). Decrease in pyoverdine production was observed when $100 \mu \text{M} \text{Cd}^{2+}, \text{Fe}^{3+}, \text{Cu}^{2+}$,
Mn$^{2+}$, Ni$^{2+}$, Eu$^{3+}$ and Tb$^{3+}$ were supplemented in the culture media (Braud et al. 2010) and in these experiments all the metals were added at the starting of the cultures.

It has been seen that iron siderophore production can be either activated or repressed by many metals depending on their concentrations. Therefore, studies should be carried out to test the large variety of metal concentrations to find out their roles in bringing the regulation of siderophores production in more elaborative ways. It is still very unclear that how other metals except iron can stimulate iron production.

One of the possibilities is that the concentration of free siderophores in the medium is reduced in the presence of other metals which causes complex formation. Such reduction in the siderophore concentration can bring activation of additional siderophores into the media. However, it was observed that the pyoverdine concentration in an overnight succinate media was 120µM which is a concentration which is much higher than 10µM of metal concentration added in the culture (Braud et al. 2009b) which indicates that the siderophore concentration was not decreased in this experimental setup. Further it was revealed that all metals (Cr$^{2+}$, Cu$^{2+}$, Ga$^{3+}$, Ni$^{2+}$, Al$^{3+}$, Mn$^{2+}$) increasing pyoverdine production were able to inhibit uptake or incorporation of pyoverdine-Fe$^{55}$ in P. aeruginosa via pyoverdine pathway (Braud et al. 2009b). The pyoverdine metal-complexes interacts with FpvA present at the cell surface which further activates FpvA/PvdS signalling cascade this mechanism does not involves uptake of any metal for siderophore production. Pyochelin biosynthesis requires cytoplasmic interactions between PchR and pyochelin-Fe which can be lethal for cell if the metal is toxic this can be a reason why this no metal is known to activate the production of this siderophore weather in iron-supplemented or limited conditions. The stimulation of other siderophores by metals other than iron has been observed for schizokinen in B. megaterium, azotochelin in A. vinelandii and pyoverdine in P. aeruginosa which further raises the question of the objective of such a process.
1.9 Other nutrients solubilised by siderophores

1.9.1 Solubilisation of zinc by siderophores

Pseudomonads have the ability to produce small molecules that are metal chelator known as
pyridine-2,6-dithiocarboxylic acid (pdtc) which has the ability to bind with Fe and therefore
known as siderophores (Ockels et. al., 1978). Because of the soft character of the sulphur
donor atoms present in the molecule of pdtc it has the ability to form complex with a variety
of soft metals which has high affinity for Fe$^{2+}$ than Fe$^{3+}$. It is also known to form complex with
zinc (Marc S. Cortese & Crawford 2002). It was first demonstrated in Pseudomonas putida
that pdtc has the ability for biological transport of the zinc ions (Lewis et. al., 2004). The
complex of zinc-pdtc is recognized and further transported through the outer membrane and
inner membrane receptors present in the pdtc utilization mechanism but the transportation
of zinc-pdtc is less efficient as compared to iron-pdtc complex (Leach, Morris & Lewis 2007).

1.9.2 Absorption of manganese by siderophores

Manganese is another important nutrient and bacterial manganese metabolism leads to the
global redox cycle of the element (Tebo et al. 2004). Mn$^{2+}$ is able to solubilize in water but
there are other forms which are highly oxidized and is deposited in the environment in the
form of Mn$^{3+}$ and Mn$^{4+}$ oxides. Many microorganisms are capable of catalysing the oxidation
of Mn$^{2+}$ but it is uncertain whether it has any biological function (Spiro TG 2012). The oxidation
bought through enzyme reactions is performed through one- electron transfer which is able
to contribute enough soluble forms of Mn$^{2+}$ intermediates but this can occur only in suboxic
environment (Francis & Tebo 2001) (G. J. Brouwers 2000). The manganese oxidizing bacteria
which are mostly studied are fluorescent pseudomonads (Parker et al. 2014). The
fluorescence is due to formation of pyoverdine siderophores. These siderophores brings
manganese metabolism which results in formation of Mn-pyoverdine complexes.
Pseudomonas putida MnB1 which is a Mn-oxidising strain is known to produce PVD$_{MnB1}$
(Webb et al. 2005). It supports all the properties except those of siderophores and has 1000
folds greater binding property for Mn$^{3+}$ than for Fe$^{3+}$. In addition to Mn$^{3+}$-siderophore
complex the investigation was done for other pyoverdines and rhizoferrin where it was found
that these have greater affinity for Mn$^{3+}$ as compared to Fe$^{3+}$(Harrington et al. 2012).
1.9.3 Interaction with molybdenum or vanadium

Some bacterial strains are known to produce nitrogenases with iron in the form of cofactor, whereas others use molybdenum or vanadium as their cofactors (Seefeldt, Hoffman & Dean 2009) (Robert L. Robson 1986) (Müller A 1992). The diazatroph Azotobacter vinelandii which is a nitrogen-fixing bacteria produces aminochelin which is a catecholamine siderophore coordinates with iron and molybdenum (Page et. al.,1988). Through aminochelin, A.vinelandii was able to absorb molybdenum from the silicates samples (Zerkle et al. 2011). The siderophores produced under limiting conditions of Mo and V forms stable complexes with them (Bellenger et al. 2008). A.vinelandii are reported to produce siderophores like pyoverdine known to be azotobactin that contains metal-binding motifs present in those of siderophores such as hydroxamate, catecholate, α-hydroxycarboxylate. Azotobactin forms complexes with vanadate and molybdate like protochelin and azotochelin which are further transported into the bacteria (Bellenger et al. 2011).

1.9.4 Interaction with copper

Since Cu$^{2+}$ are similar to Zn$^{2+}$ than Fe$^{3+}$ and siderophores that form complexes with ferric ions which have hard ligands will not be able to form complexes with cupric ions as they require their ligands forms different geometries, which suggests lack of interactions between copper ions and the siderophores that are pre-organised to form complexes with Fe$^{3+}$ but siderophores having soft donor atoms are able to bind with copper. Therefore, these interactions are believed to have significant biological purpose. The above mentioned metal binding studies of pdtc siderophores can identify copper as a ligand (Cortese MS 2002). Earlier studies showed that metal complexes of pdtc can dehalogenate carbon tetrachloride (Dybas et.al.,1995) (Dybas MJ 1998) and the most active was founded to be the cupric pdtc complex (Sebat et al. 2001). Pdtc as a siderophore is not used by bacteria to increase the intracellular concentration of copper (Leach & Lewis 2006). The role of pseudomonal siderophores in copper regulation was obtained through observing the changes in the transcription of P. aeruginosa after exposure to the high concentration level of copper (Teitzel et al. 2006).
1.10 **Aim**

To isolate and identify a potential PGPB candidate towards canola and establish its mode of action for how the bacterial isolate enhances plant growth

1.11 **Objectives**

1. Isolate potential PGPB candidates from soil samples taken from key canola growing areas in Victoria
2. Identify PGPB isolates by screening for known traits that enhance plant growth and validate by inoculating on canola plants and observing increased dry root and shoot biomass
3. Determine mode of action for bacterial isolate to enhance canola growth
2.0 Materials and methods

2.1 Identification of the plant growth promoting bacterial (PGPB) isolate *Bacillus coagulans* DUS-M6, isolated from soil samples collected from Maryborough in Central Victoria

Isolated colonies, from soil samples diluted in 1 x phosphate buffered solution (PBS) and streaked out on solid TY medium, were screened for traits known to promote plant growth including: siderophore production, was assessed by growing colonies on Chromeazurol S media as previously described (Louden et al., 2011); phosphate solubilisation, where colonies were grown on Pikovskaya media as previously described (Nautiyal, 1999); IAA production, cells were grown in glucose peptone broth and Salkowski reagent used to give a colorimetric-based quantification of IAA production as previously described (Sarwar and Kremer, 1995); and ACC deaminase activity, where cells were grown in Dworkin and Foster medium as previously described (Dworkin and Foster, 1958) and ACC deaminase activity detected as previously described (Penrose and Glick, 2003). Bacterial isolates positive for all of the plant growth promotion tests were identified by sequencing (AGRF, Melbourne) gel purified polymerase chain reaction (PCR) products of their 16s rDNA gene using 27F and 1492R primers following previously described methods (Hudek et al., 2015). Based on the outcomes from these analyses, the putative plant growth promoting *Bacillus coagulans* DUS-M6 was identified where the 16s rDNA sequence obtained through routine sequencing of PCR amplified 16s rRNA from the initial “unknown” bacterial cells were BLAST searched against 16S rRNA database ([https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)) with a sequence identity of >98.5% and highest identity determined to be shared with *Bacillus* species, in particular *B. coagulans*.

2.2 *Bacillus coagulans* DUS-M6 culture conditions

*Bacillus coagulans* DUS-M6 cells were cultured in liquid tryptone-yeast extract (TY) medium at 28 °C in a shaking incubator (180 rpm) for 24 h, or, streak plated on TY agar (1.5%) and grown for 24 h at 28 °C. Cell stocks were stored frozen at -80 °C in TY with 20% added glycerol. Cell numbers were standardised for all experiments to an optical density of 0.15 at a wave length of 600 nm (OD\(_{600}\)), which correlates to ~1 x 10\(^3\) CFU/ml) confirmed by Miles and Misra plate counts unless specified otherwise. (Fox et al., 2011) (Miles et al., 1938)
2.3 Inducing siderophore production by *B. coagulans* DUS-M6

*Bacillus coagulans* DUS-6 was grown on a sodium succinate medium containing (per liter) 6.0 g of K$_2$HPO$_4$, 3.0 g of KH$_2$PO$_4$, 1.0 g of (NH$_4$)$_2$SO$_4$, 0.1 g of MgSO$_4$.7H2O, and 4.0 g of succinic acid. The pH was adjusted to 7.0 by adding 1N NaOH solution prior to sterilization. The medium was dispensed into 500 ml Erlenmeyer flasks, each of which contained 100 ml of medium. The flasks were then inoculated, at a seed rate of 1.0%, with exponential-phase cells grown in the same medium. The cultures were incubated at 25 °C and 200 rpm in a New Brunswick model Innova 4000 shaker-incubator for 48 h. Batches were pooled and centrifuged at 10,000 rpm and 3 g for 10 min at 48 °C. The resulting supernatant was membrane filtered (pore size, 0.25 mm; Amicon) to yield a cell-free solution of crude pyoverdines. The amount of pyoverdine produced was estimated by measuring the absorbance of the supernatant at a wavelength of 400 nm.

2.4 Purification of siderophore produced by *B. coagulans* DUS-M6 using High performance liquid chromatography (HPLC)

The further purification and confirmation of siderophore production by *B. coagulans* DUS-M6 was performed using HPLC (Dionex UltiMate 3000 Dioade Array and Multiple-Wavelength detector running through the computer program Chromeleon v7.2) with an Acclaim 120 C18 3 µM 120 Å 2.1 x 150 mm column (Prod. No. 059130) and detecting the spectra of the molecules between 200 and 500 nm following previously described methods (Bultreys, 2003). For the mobile phases, solution A was 17 mM NaOH-acetic acid buffer (pH 5.3) and solution B was 100% acetonitrile. The HPLC program previously described by (Bultreys *et al* 2003) was followed where: (flow rate, 1 ml/min): 100% solution A, 1 min; from 100% solution A to 97% solution A, 2 min; 97% solution A, 9 min; and from 97% solution A to 30% solution A, 25 min. HPLC program 2 (flow rate, 0.9 ml/min) was as follows: 100% solution A, 8 min; from 100% solution A to 98% solution A, 2 min; 98% solution A, 10 min; from 98% solution A to 95% solution A, 5 min; from 95% solution A to 30% solution A, 15 min; and 30% solution A, 5 min. The unknown siderophore samples from *B. coagulans* DUS-M6 were compared against a standard sample (5 mg/mL) of the siderophore pyoverdine (Sigma, Cat. No. P8124).
2.5 Growth conditions for *B. napus* L. (canola)

In three independent experiments (triplicates), three surface sterilised *B. napus* seeds (Pioneer Hybrid 45Y66 cultivar, donated by Elders, Geelong, Australia) were germinated and grown in 20 mL of natural gravel (5 – 15 mm aggregate size) that had been treated to remove all bioavailable trace metals as follows: One litre of gravel was rinsed 5 times in 2 L of deionised water feed through a 18 mOhm (Milli-Q) water filtration system, then washed 3 times in 2 L 6 M HCl, rinsed 3 times in 2 L of deionised water feed through a 18 mOhm (Milli-Q) water filtration system, water drained off, autoclaved 2 times at 121°C for 30 min to sterilise and dried in an oven at 60°C for 4 days. A volume of 20 mL of deionised water feed through a 18mOhm (Milli-Q) water filtration system, purified water containing 25 µM either Fe, Zn, Co or Cu, or, 20 mL of Hoagland and Arnon (Hoagland & Arnon 1950) media was added to 40 mL of the dried sand with three canola seeds placed 1 cm within the gravel and grown for 14 days at 22 °C in a phytotron with a 16:8 h light: dark cycle. For plants inoculated with *B. coagulans*, ~1 x10³ cells per mL (correlating to an optical density of ~0.15) were added to the 20 mL of liquid at the initial planting stage. For treating plants with purified siderophores, 50 mg of purified freeze-dried powder was added to the liquid at the initial planting stage.

2.6 Quantification of metal (Fe, Co, Cu, Zn and Mn) levels in *B. napus* plants

Concentrations of metals including Ni, Zn, Co, Fe and Mn in *B. napus* roots and shoots grown in natural gravel after 14 days were determined using inductively coupled plasma mass spectrometry (ICP-MS). Dry roots and shoots were digested using 2 mL concentrated aqua regia (4 HCl:1HNO₃) for 3 days at room temperature. Samples were diluted to 1% aqua regia for analyses with rhodium used as the internal standard. The certified 1573a tomato leaf standard (National institute of standards and technology) was used as a reference.

2.7 Identification and retrieval of putative catalase gene sequences for *B. napus* and *B. coagulans* DUS-M6

The complete genome sequence of *B. napus* (NCBI Reference Sequence assembly accession: GCF_000686985.1) was screened for sequences with high identity to the catalase proteins from a range of plants including, *Arabidopsis thaliana*, *Zea mays* L. and *Nicotiana tabacum* using the Basic Local Alignment Search Tool (BLAST) (Boldt & Scandalios 1997; Frugoli et al. 1998; Mhamdi et al. 2010; Scandalios 1997). Primers were designed for qRT-PCR
experiments using the retrieved nucleotide sequences for the putative catalases in *B. napus*: 

- **NRAMP1** (accession number 013821469.1)
- **NRAMP2** (accession number 013842817.1)
- **NRAMP3** (accession number 013817363.1)
- **NRAMP4** (accession number 013830755.1)
- **NRAMP5** (accession number 013855086.1)
- **NRAMP6** (accession number 013836580.1)
- **CT1** (accession number 013801650.1)
- **CT2** (accession number 013791453.1)
- **ZIP2** (accession number 013858133.1)
- **EIN2** (accession number 013876976.1)
- **YSL1** (accession number 013816048.1)
- **YSL2** (accession number 013816048.1)
- **YSL3** (accession number 013811337.1)
- **YSL5** (accession number 013829412.1)
- **YSL6** (accession number 013854705.1)
- **YSL7** (accession number 013866982.1)
- **YSL8** (accession number 013873877.1)

### 2.8 Extraction and purification of RNA, and cDNA synthesis

For *B. napus* samples, extraction and purification of RNA was performed using 100 mg (wet weight) of freshly harvested plant tissue frozen in liquid nitrogen, ground to a paste with a mortar and pestle, along with 1 µL of Protector RNase inhibitor. Subsequent isolation and purification were achieved using a QIAGEN RNeasy Plant Mini Kit, following the previously described modified protocol (Hudek *et al.* 2009; Hudek *et al.* 2017; Song *et al.* 2014).

Following extraction, the RNA from *B. napus* was further purified using the Ambion DNA-free kit according to the manufacturer’s protocol (Ambion, Australia) with 1 µL of Protector RNase inhibitor added to the RNA after the Ambion DNA-free treatment. Total RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Australia). Complementary-DNA (cDNA) synthesis was achieved using the High-capacity cDNA Reverse Transcription Kit as per the manufacturer’s protocol (Thermo Scientific, Australia).

### 2.9 Measurement of changes to relative mRNA levels for metal transport genes in *B. napus* using quantitative real-time PCR (qRT-PCR)

Quantitative real-time-PCR (qRT-PCR) was used to investigate the transcriptional response of the NRAMP1, NRAMP2, NRAMP3, NRAMP4, NRAMP5, NRAMP6, CT1, CT2, ZIP2, EIN2, YSL1, YSL2, YSL3, SRT1, YSL5, YSL6, YSL7, YSL8 in *B. napus*. Housekeeping primers targeting *actin-2*
(act2) in *B. napus* were also produced. Primers were designed using Primer Express (v2.0 for Windows 2000, Applied Biosystems, Australia) and tested using established methods (Hudek *et al.* 2009; Hudek *et al.* 2017; Song *et al.* 2014) (Table 1). Primer binding efficiencies were established using 1, 2, 4 and 8 µg mL$^{-1}$ of template cDNA as a control, then comparing change in cycle-time (ΔCt) of amplification against increasing cDNA concentration. The qRT-PCR was completed according to the manufacturers protocol using 1 × SYBR Green Master Mix (Applied Biosystems, Australia), 20 ng of cDNA template, and 0.3 µM of forward and reverse primers. Quantitative real-time PCR analyses were conducted using Applied Biosystems 7500 Real Time PCR system and Biosystems 7500 SDS software (Applied Biosystems, Australia). Stages of the qRT-PCR were followed as previously described (Hudek *et al.* 2009; Hudek *et al.* 2017; Song *et al.* 2014): Stage 1. 50 °C for 2 min, Stage 2. 95 °C for 10 min, Stage 3. 95 °C for 15 s followed by 60 °C for 10 min, repeated for 40 cycles and Stage 4 (dissociation step) 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

### 2.10 Statistical methods

Statistical analyses were based on normally distributed data produced from three independent replicate experiments of 3 plants each. The statistical program IBM SPSS Statistics 25 (for Windows) was used for all statistical analysis. Probability plots (P-P Plots) were produced for all data sets to test for normal distribution. Multiple comparison of means was determined by a one-way analysis of variance (one-way ANOVA) and Tukey’s honest significant difference test for the plant and bacterial growth, quantification of metals using ICP-MS and qRT-PCR data sets. All statistical analyses were tested against the probability value (p-value) of <0.05.

**Table 1.** Primers for amplification of catalase genes in *B. napus*. Forward and reverse primers were denoted by ‘F’ and ‘R’ respectively.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’ orientation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRAMP1 F</td>
<td>GTGCCGCTTTGGTGATTCA</td>
</tr>
<tr>
<td>NRAMP1 R</td>
<td>TCTACAGTGCTCAGCCAATGTTT</td>
</tr>
<tr>
<td>NRAMP2 F</td>
<td>TCGGTAGTGCGATAGCGATTC</td>
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<tr>
<td>Gene</td>
<td>Forward Sequence</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>NRAMP2</td>
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</tr>
<tr>
<td>NRAMP3</td>
<td>CCTTTGCTCGTGGCATTAATT</td>
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<tr>
<td>YSL3</td>
<td>CAACCCGCATCCGACAA</td>
</tr>
<tr>
<td>SRT1</td>
<td>CCAAAATTACCATTCCCCCTCAAATA</td>
</tr>
<tr>
<td>SRT1</td>
<td>TTTGCAAGCACGCCCTCCCTT</td>
</tr>
<tr>
<td>YSL5</td>
<td>CATACCTCTTTGGCATGAGTAAC</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td><strong>YSL5 R</strong></td>
<td>TCCAACCCAAAGAAGGATCCT</td>
</tr>
<tr>
<td><strong>YSL6 F</strong></td>
<td>TGGTGCTGACTATCCTGGGAAT</td>
</tr>
<tr>
<td><strong>YSL6 R</strong></td>
<td>ACAAAACAAAAACCAATCATCCA</td>
</tr>
<tr>
<td><strong>YSL7 F</strong></td>
<td>CGTCGGTCTATACAAGCAGTTC</td>
</tr>
<tr>
<td><strong>YSL7 R</strong></td>
<td>GAAATGGTACGGTGTGTTGATGGT</td>
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</tr>
<tr>
<td><strong>YSL8 R</strong></td>
<td>AACTCCCCAACACTCTCATTG</td>
</tr>
</tbody>
</table>

3.0 Results

3.1 Identification of *Bacillus coagulans* DUS-M6 based on classical plant growth promoting traits

Single colonies were obtained by streaking-out soil samples on solid TY agar medium from soil samples collected from Maryborough in Victoria. Colonies were purified and screened for the following traits: siderophore production (Fe solubilisation), ability to solubilise phosphate, production of IAA, ACC deaminase production and increased plant growth when inoculated on *B. napus* after 14 days (Table 2). Using 16S rRNA sequencing, one of the isolates that had all these PGP traits and increased *B. napus* growth was identified as being *Bacillus coagulans*. The identified *B. coagulans* was given the strain name Deakin University Strain – Maryborough 6 (DUS-M6).

Table 2: *B. coagulans* DUS-M6 characteristics, production of iron solubilising siderophores, ability solubilise phosphate, ACC deaminase activity and production of IAA.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Siderophore production</th>
<th>Phosphate solubilization</th>
<th>IAA production</th>
<th>ACC deaminase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. coagulans</em> DUS-M6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.2 Detection of pyoverdine like siderophore peptide of the isolated strain *B. coagulans* DUS-M6 through High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was used to further identify the metal-chelating peptides (siderophores) produced by *B. coagulans* DUS-M6 in comparison with a known standard pyoverdine peptide (Sigma Cat. no. PB-142) produced by *Pseudomonas fluorescens*. Similarities between the profile of the chromatograms and the peaks detected at 405 nm for the peptide produced by *B. coagulans* DUS-M6 and pyoverdine peptide standard, indicate that the *B. coagulans* DUS-M6 produces a pyoverdine-like peptide (Fig 4A and Fig 4B).
Fig. 4. Detection of a pyoverdine-like peptide (siderophore) produced by B. coagulans DUSM6. A known standard pyoverdine peptide (PB-124) from *Pseudomonas fluorescens* (A) was used to compare peptides produced by *B. coagulans* DUS-M6 (B). The peptides were detected at a UV wavelength of 405 nm and with a similarity in peptide profiles and a conserved retention time of ~3 min and 35 s (from 11 min 25 s to 15 min) observed for both peaks.
3.3 Effect of inoculating *B. napus* (canola) with *B. coagulans* DUS-M6

To determine if *B.coagulans* DUS-M6 was capable of enhancing canola growth, it was inoculated onto *B. napus* plants and assessed after 14 days. The inoculation of plants with the isolated strain (at 0.15 OD<sub>600nm</sub>, approximately 1 x 10<sup>3</sup> CFU/mL) in the pots for 14 days significantly increase plant growth, based on increased levels of dry plant root and shoot biomass compared to uninoculated control plants (Fig 5). The effect of the inoculation of *B. coagulans DUS-M6* on enhancing growth, was also assessed for the effect the addition of metals:: uninoculated plants ± metal ions (Fe, Zn, Cu, Co or Mn); plants inoculated with *B.coagulans* DUS-M6 ± Fe, Zn, Cu, Co or Mn; plants treated with purified siderophores produced by *B.coagulans* DUS-M6 ± Fe, Zn, Cu, Co or Mn; and plants treated with the commercial pyoverdine peptide ± Fe, Zn, Cu, Co or Mn (Fig 5). For all treatment groups (± Fe, Zn, Cu, Co or Mn) the inoculation of plants with *B. coagulans* DUS-M6 significantly increased root biomass by 2-6 mg in comparison with respective uninoculated controls (Fig 5A).

For all treatment groups (± Fe, Zn, Cu, Co or Mn) the addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased root biomass by 2-6 mg in comparison with respective uninoculated controls (Fig 5A). The addition of the commercial pyoverdine siderophore increased root biomass by 2-4 mg for plants with no added metals, added Fe, Zn and Cu in comparison with respective uninoculated controls (Fig 5A). In comparison with the commercial siderophore (pyoverdine), the siderophore-like peptide
produced by *B. coagulans* DUS-M6 significantly increased root biomass for the control
treatment and the added Fe, Cu, Co and Mn treatments by 2-3 mg (Fig 5A). Further to these
findings, the siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly
increased plant root biomass for the Fe, Cu and Mn above all other treatment groups,
including the plants inoculated with *B. coagulans* DUS-M6 cells (Fig 5A).

The inoculation of plants with *B. coagulans* DUS-M6 cells significantly increased the plant
shoot biomass for plants treated with added Fe and Mn, compared to the uninoculated
control plants and all other treatments (Fig 5B). Interestingly, for plants treated with added
cobalt (Co), the addition of the siderophore-like peptide produced by *B. coagulans* DUS-M6,
or the addition of the commercial siderophore (pyoverdine) significantly reduced shoot dry
biomass compared to uninoculated control plant shoots (Fig 5B).
Fig. 5. Effect of inoculation of *B. napus* germination and growth after 14 days, based on dry root (A) or dry shoot (B) biomass for plants treated with Bacillus DUS-M6 cells, treated with purified siderophore produced by *Bacillus coagulans* DUS-M6 or treated with siderophore (pyoverdine) standard in the presence or absence of added Fe, Zn, Cu, Co or Mn. Significant difference (*p*<0.05) between the uninoculated control and the different treatment groups is denoted by superscript letters (a, b, c), with statistically similar (*p*>0.05) responses grouped by the same letter.
3.4 Inoculation of *B. napus* with *B. coagulans* DUS-M6 or the purified siderophores produced by *B. coagulans* DUS-M6 alters levels of the trace elements Fe, Zn, Co, Cu and Mn in plants

Using ICP-MS, the effect of inoculation with *B. coagulans* DUS-M6, the addition of purified siderophore-like peptide produced by *B. coagulans* DUS-M6, or, the addition of the commercial pyoverdine had on metal uptake in *B. napus* was measured after 14 days of growth (Table 2 and 3). The accumulated levels of Zn, Co and Mn were significantly higher in inoculated plants roots, compared with uninoculated plants roots by approx. 0.3, 0.2 and 2 nM per mg of dry plant root tissue, respectively (Table 3). Inoculation of plants with *B. coagulans* DUS-M6 significantly increased levels of Zn and Mn in plants roots beyond control levels for all other treatments (Table 3). In plant roots, the addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe, Co, Cu and Mn accumulation in comparison to controls by approx. 0.7, 0.2, 0.3 and 0.4 nM per mg of dry plant root tissue, respectively (Table 3). The addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe, Co and Cu levels above all other treatments (Table 3). The addition of the commercial pyoverdine increased Fe and Co uptake significantly compared to control plants, by approx. 0.2 and 0.1 nM per mg of dry plant root tissue, respectively, but had less effects overall compared to the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 (Table 3).

The accumulated levels of Fe, Zn and Cu were significantly higher in inoculated plants shoots, compared with uninoculated plants shoots by approx. 0.9, 0.4 and 0.1 nM per mg of dry plant shoot tissue, respectively (Table 4). In plant shoots, the addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe, Zn, Cu and Mn
accumulation in comparison to controls by approx. 1.7, 0.3, 0.2 and 0.5 nM per mg of dry plant shoot tissue, respectively (Table 4). The addition of the purified siderophore-like peptide produced by B. coagulans DUS-M6 significantly increased Fe and Mn uptake in plant shoots above levels of all other treatment groups (Table 4). The commercial pyoverdine significantly increased Fe and Zn levels in plant shoots by 0.9 and 0.3 nM per mg of dry plant shoot tissue, respectively (Table 4). For Co, the levels taken-up into plant shoots were significantly lower for inoculated plants or when the purified siderophore-like peptide produced by B. coagulans DUS-M6 or the commercial pyoverdine was added, with Co levels reduced by 0.5 1.5 and 0.5 respectively, with the purified siderophore-like peptide produced by B. coagulans DUS-M6 significantly reducing Co levels in comparison to all other treatment groups (Table 4).
Table 3. Effect of *B. coagulans* DUS-M6 inoculation or addition of purified *B. coagulans* DUS-M6 siderophore on metal (Fe, Zn, Co, Cu or Mn) levels (nM per mg of dry plant root biomass) accumulated in *B. napus* after 14 days.

<table>
<thead>
<tr>
<th></th>
<th>Uninoculated root</th>
<th><em>B. coagulans</em> DUS-M6 root</th>
<th><em>B. coagulans</em> DUS-M6 siderophore root</th>
<th>Pyoverdine Std. root</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fe</strong></td>
<td>0.88 ± 0.05</td>
<td>0.91 ± 0.09</td>
<td>1.41 ± 0.38</td>
<td>1.03 ± 0.21</td>
</tr>
<tr>
<td><strong>Zn</strong></td>
<td>0.59 ± 0.04</td>
<td>0.83 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49 ± 0.08</td>
<td>0.57 ± 0.08</td>
</tr>
<tr>
<td><strong>Co</strong></td>
<td>10.3 ± 0.07</td>
<td>0.36 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Cu</strong></td>
<td>0.31 ± 0.05</td>
<td>0.34 ± 0.05</td>
<td>0.59 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td><strong>Mn</strong></td>
<td>1.45 ± 0.18</td>
<td>3.27 ± 0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.84 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.12 ± 0.17</td>
</tr>
</tbody>
</table>
Table 4. Effect of *B. coagulans* DUS-M6 inoculation or addition of purified *B. coagulans* DUS-M6 siderophores on metal (Fe, Zn, Co, Cu or Mn) levels (nM per mg of dry plant shoot biomass) accumulated in *B. napus* after 14 days.

<table>
<thead>
<tr>
<th></th>
<th>Uninoculated shoot</th>
<th><em>B. coagulans</em> DUS-M6 shoot</th>
<th><em>B. coagulans</em> DUS-M6 siderophore shoot</th>
<th>Pyoverdine Std. shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fe</strong></td>
<td>0.89 ± 0.06</td>
<td><strong>1.77 ± 0.71</strong>a</td>
<td>2.50 ± 0.25b</td>
<td>1.78 ± 0.22a</td>
</tr>
<tr>
<td><strong>Zn</strong></td>
<td>0.32 ± 0.09</td>
<td>0.87 ± 0.10a</td>
<td>0.73 ± 0.044</td>
<td>0.63 ± 0.03b</td>
</tr>
<tr>
<td><strong>Co</strong></td>
<td>1.99 ± 0.21</td>
<td>1.02 ± 0.15a</td>
<td>0.43 ± 0.06b</td>
<td>0.93 ± 0.27a</td>
</tr>
<tr>
<td><strong>Cu</strong></td>
<td>0.78 ± 0.09</td>
<td>0.90 ± 0.08a</td>
<td>0.95 ± 0.06a</td>
<td>0.76 ± 0.08</td>
</tr>
<tr>
<td><strong>Mn</strong></td>
<td>1.48 ± 0.23</td>
<td><strong>1.37 ± 0.40</strong></td>
<td><strong>2.02 ± 0.18</strong></td>
<td>1.57 ± 0.38</td>
</tr>
</tbody>
</table>
3.5 Changes to trace element homeostasis mechanisms in *B. napus* grown in different metal treatments (no metals, Fe, Zn, Co, Cu or Mn) and treated with *B. coagulans* DUS-M6 or the purified siderophores produced by *B. coagulans* DUS-M6

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine if inoculation of *B. napus* with *B. coagulans* DUS-M6 or the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore altered the expression of metal sensing and transporting genes, based on changes in relative mRNA level standardised against the constitutently expressed *actin*-2 house-keeping gene (Table 5 and 6). Primers were designed specifically for the qRT-PCR experiments using the software Primer Express (v2.0 for Windows 2000, Applied Biosystems, Australia), with all primers having a Tm of ~62 °C and product size of ~150 bp for consistency, enabling comparison between quantification of a consistent fluorescent signal produced by each genes product. This is also ensures that comparisons are consistent between the different genes, with respect to the comment housekeeping gene (2-Actin).

Inoculation of *B. napus* with *B. coagulans* DUS-M6 or the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore increased the relative mRNA levels for the *NRAMP1* gene in roots from plants grown without added metals or with added Fe (Table 5). In the roots of plants treated with Co, Cu or Mn, inoculation or the addition of the *B. coagulans* DUS-M6 siderophore, *NRAMP1* relative mRNA levels were generally lower in comparison with those of uninoculated plants (Table 5). For Zn treated plants, expression of *NRAMP1* was reasonably constant for the different treatment groups in plant roots (Table 5). The addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore reduced relative mRNA levels of *NRAMP2* in roots from plants grown without added metals (Table 5). In the roots of plants treated with...
Fe, inoculation or the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore increased the relative mRNA levels for the *NRAMP2* compared to the uninoculated control (Table 5). Expression of *NRAMP2* was increased in roots from plants treated with the *B. coagulans* DUS-M6 pyoverdine-like siderophore and either Zn or Mn (Table 5). In roots from plants treated with Co or Cu, *NRAMP2* expression was reasonably constant for the different treatment groups (Table 5). The inoculation of plants without added metals had the most profound effect of *NRAMP3* relative mRNA levels in roots compared with all other treatment groups (Table 5). *NRAMP3* expression was reasonably constant for the different treatment groups and metal treatments in plant roots (Table 5). The inoculation of plants without added metals had the most profound effect of relative mRNA levels of *NRAMP3* and *NRAMP6* in roots compared with all other treatment groups (Table 5). *NRAMP3* expression was reasonably constant for the different treatment groups and metal treatments in plant roots (Table 5). The treatment of plants with added Cu and *B. coagulans* DUS-M6 pyoverdine-like siderophore increased the relative mRNA levels of *NRAMP4*, whereas other treatment groups and metal treatments were relatively constant in their effect on its expression (Table 5). In roots from plants treated with added Cu, the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore reduced *NRAMP5* relative mRNA levels compared to the uninoculated control and inoculated plants (Table 5).

Relative mRNA levels of CT1 and CT2 were relatively constant for the different treatment groups and metal treatments in plant roots. The addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore increased relative mRNA levels of *CT2* in roots from plants treated with Fe (Table 5). For *Zip1*, in roots from plants treated with Co or Mn, inoculation increased relative mRNA levels compared to the uninoculated plants (Table 5). The addition
of the *B. coagulans* DUS-M6 pyoverdine-like siderophore showed to increase *Zip1* expression in roots from plants treated with Co (Table 5). Relative mRNA levels of *EIN2* were relatively constant across the different treatment groups, apart from in roots from inoculated plants without added metals, where the relative mRNA levels were reduced by 50% (Table 5). In plant roots, relative mRNA levels of *SRT1* were constant for the different treatment groups and metals treatments (Table 5).

Generally, relative mRNA levels for *YSL1* in roots from plants were increased by inoculation with *B. coagulans* DUS-M6 (Table 5). Inoculation of *B. napus* with *B. coagulans* DUS-M6 increased the relative mRNA levels for the *YSL1* in roots from plants grown without added metals; a similar, but less exaggerated trend was also observed in roots of plants treated with *B. coagulans* DUS-M6 pyoverdine-like siderophore (Table 5). Relative mRNA levels of *YLS2* and *YSL3* were constant across treatment groups and for the metal treatments (Table 5). The relative mRNA levels of *YSL5* were increased in roots from plants without added metals and inoculated or treated with *B. coagulans* DUS-M6 pyoverdine-like siderophore (Table 5). In roots from plants treated with Fe, Zn, Co, Cu or Mn *YSL5* relative mRNA levels were reduced by inoculation or the addition of *B. coagulans* DUS-M6 pyoverdine-like siderophore (Table 5). The expression of *YSL6* fluctuated across the treatment groups and metals treatments. In roots from inoculated plants treated with no added metals, Fe, Co, Cu or Mn relative mRNA levels of *YLS6* were 10% of the levels for uninoculated plants; whereas for inoculated plants treated with Zn *YSL6* expression was increased by 60% above the uninoculated control (Table 5). In roots from plants treated with treated with *B. coagulans* DUS-M6 pyoverdine-like siderophore and Zn *YSL6* expression was also increased by above the uninoculated control by ~60% (Table 5). Relative mRNA levels of *YSL7* were relatively constant across the different
treatment groups, apart from in roots from inoculated plants or plants treated with without added metals, where the *B. coagulans* DUS-M6 pyoverdine-like siderophore where relative mRNA levels were increased by up to 25% in comparison with the uninoculated control (Table 5).

Relative mRNA levels of *NRAMP1* in the shoots from inoculated plants or plants treated with *B. coagulans* DUS-M6 pyoverdine-like siderophore were generally within 10% of the uninoculated control for the no added metal and metal treatments (Table 6). In shoots from plants treated with Co and Cu, inoculation reduced *NRAMP1* expression by over 15% and for Cu the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore also reduced NRAMP1 expression by over 15% (Table 6). In shoots of inoculated plants, *NRAMP2* expression levels were 40% lower compared to the uninoculated control (Table 6). The inoculation of plants and treatment with Zn, Cu and Mn increased NRAMP2 expression by 20% over the uninoculated control (Table 6). Inoculation and treatment of plants with Co reduced *NRAMP2* relative mRNA levels to just 12% of the levels in the uninoculated control plant shoots (Table 6). For plants treated with Fe, the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophore increased *NRAMP2* expression by 20% in comparison to the uninoculated control (Table 6). In shoots from plants treated with the *B. coagulans* DUS-M6 pyoverdine-like siderophore and no added metals *NRAMP2* expression levels were 20% lower than the levels of uninoculated plants. For Cu treated plants the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophores increased *NRAMP2* relative mRNA levels by 10% in comparison with uninoculated control plants (Table 6). Relative mRNA levels for *NRAMP3*, *NRAMP4* and *NRAMP5* were constant across treatment groups for each metal. In shoots of plants that were inoculated and treated with no added metals, *NRAMP3* expression was 20%
above the uninoculated control (Table 6). In shoots of plants treated with Cu and the *B. coagulans* DUS-M6 pyoverdine-like siderophores *NRAMP4* expression was 80% above the uninoculated control (Table 6). Compared to uninoculated control plants, *NRAMP6* relative mRNA levels increased by 15-25% in shoots of plants grown with no added metals and either inoculated or treated with the *B. coagulans* DUS-M6 pyoverdine-like siderophores (Table 6). For plants treated with added Fe, inoculation or the addition of the *B. coagulans* DUS-M6 pyoverdine-like siderophores increased *NRAMP6* relative mRNA levels by 10% compared to uninoculated controls (Table 6).

For all treatment groups and the Zn, Co and Cu metal treatments, *CT1* and *CT2* relative mRNA levels were generally within 10% of the levels in the uninoculated control in plant shoots (Table 5). In inoculated plants treated with Fe, *CT1* and *CT2* expression was increased by 20-30% compared to uninoculated controls (Table 6). In plants treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores and no added metals or Mn added, *CT1* and *CT2* relative mRNA levels were increased by over 20% compared with uninoculated control levels (Table 6). In plant shoots, *ZIP1* relative mRNA levels were all within 10% across all treatment groups (Table 5). Relative mRNA levels for *EIN2* were 40% lower in inoculated plant shoots treated with Fe compared levels of the uninoculated control (Table 6). For all other treatments across treatment groups, relative mRNA levels for *EIN2* were within 10% (Table 6). For all treatments across all treatment groups, relative mRNA levels for *SRT1* were within 5% of each other (Table 6).
In the shoots of plants grown without added metals and either inoculated or treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores, *YSL1* relative mRNA levels were increased by 15% compared to the uninoculated control (Table 6). For all other metal treatments across the different treatment groups relative mRNA levels for *YSL1* were within 10% (Table 6). Relative mRNA levels for *YSL2* and *YSL3* were within 10% across all metal treatments for the different treatment groups (Table 6). Compared to uninoculated controls, *YSL5* relative mRNA levels in shoots of plants either inoculated or treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores and treated with added Fe were reduced by 15%, and in shoots from plants with added Cu *YSL5* expression was reduced by ~25% (Table 6). In shoots from inoculated plants with added Mn, *YSL5* relative mRNA levels were reduced by ~30% compared to uninoculated controls (Table 6). Relative mRNA levels of *YSL6* varied greatly for the different metal treatments of the different treatment groups (Table 6). In the shoots of inoculated plants without added metals or plants treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores without added metals, *YSL6* relative mRNA levels were increased by ~90% compared to uninoculated control levels (Table 6). For the shoots of inoculated plants with added Fe, *YSL6* relative mRNA levels were 99% lower than the uninoculated controls, whereas for the *B. coagulans* DUS-M6 pyoverdine-like siderophores and Fe treated plants *YSL6* expression was 99% above the uninoculated control (Table 6). In shoots from inoculated plants treated with Zn or Mn, *YSL6* expression was reduced by 50 and 40% respectively (Table 6). In shoots from inoculated plants with added Co or plants treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores plus Co, *YSL6* expression was reduced by 80% and 30% compared to the uninoculated control, respectively (Table 6). For the shoots of inoculated plants with added Cu, *YSL6* relative mRNA levels were 94% lower than the uninoculated
controls, whereas for the *B. coagulans* DUS-M6 pyoverdine-like siderophores and Cu treated plants *YSL6* expression was 25% above the uninoculated control (Table 6).

In the shoots of plants treated with Mn and either inoculated or treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores, *YSL7* relative mRNA levels were increased by over 30% compared to the uninoculated control (Table 6). In the shoots of plants treated with Fe or Cu and either inoculated or treated with *B. coagulans* DUS-M6 pyoverdine-like siderophores, *YSL7* relative mRNA levels were reduced by 30% compared to the uninoculated control (Table 6). In shoots from inoculated plants without added metals, *YSL7* expression was ~35% higher compared to levels of the uninoculated control (Table 6). In shoots from plants treated with Zn and *B. coagulans* DUS-M6 pyoverdine-like siderophores, *YSL7* relative mRNA levels were increased by ~15% (Table 6).
Table 5. Expression profile (heat map) of relative mRNA levels for *B. napus* metal transporters in uninoculated plant roots (+/- metal treatment), inoculated plant roots (+/- metal treatment) and roots from plants treated with *B. coagulans* DUS-M6 siderophore (+/- metal treatment). The greener colouring denotes the lower values (based on calculated relative mRNA levels standardised against the actin-2 house-keeping gene) for the specific gene (row) with the transition from green to yellow to red indicating increases in relative mRNA levels (where dark red denotes the highest relative mRNA levels).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative mRNA levels in roots of uninoculated plants</th>
<th>Relative mRNA levels in roots of plants inoculated with <em>B. coagulans</em> DUS-M6 siderophore</th>
<th>Relative mRNA levels in roots of plants treated with <em>B. coagulans</em> DUS-M6 siderophore</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No metals</td>
<td>Fe</td>
<td>Zn</td>
</tr>
<tr>
<td>NRAMP1</td>
<td>1.50</td>
<td>1.52</td>
<td>1.63</td>
</tr>
<tr>
<td>NRAMP2</td>
<td>1.05</td>
<td>0.65</td>
<td>0.66</td>
</tr>
<tr>
<td>NRAMP3</td>
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<td>1.62</td>
<td>1.60</td>
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<tr>
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<td>1.54</td>
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<td>NRAMP6</td>
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<td>0.97</td>
</tr>
<tr>
<td>CT1</td>
<td>0.79</td>
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<td>0.83</td>
</tr>
<tr>
<td>CT2</td>
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<td>1.20</td>
<td>1.07</td>
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<td>ZIP1</td>
<td>1.13</td>
<td>1.09</td>
<td>1.13</td>
</tr>
<tr>
<td>EIN2</td>
<td>0.83</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>SRT1</td>
<td>0.87</td>
<td>0.88</td>
<td>0.87</td>
</tr>
<tr>
<td>YSL1</td>
<td>1.79</td>
<td>1.59</td>
<td>1.64</td>
</tr>
<tr>
<td>YSL2</td>
<td>2.15</td>
<td>2.17</td>
<td>2.17</td>
</tr>
<tr>
<td>YSL3</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>YSL5</td>
<td>3.39</td>
<td>3.37</td>
<td>3.20</td>
</tr>
<tr>
<td>YSL6</td>
<td>6.58</td>
<td>3.65</td>
<td>8.81</td>
</tr>
<tr>
<td>YSL7</td>
<td>3.85</td>
<td>3.57</td>
<td>3.57</td>
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</table>
Table 6. Expression profile (heat map) of relative mRNA levels for *B. napus* metal transporters in uninoculated plant shoots (+/- metal treatment), inoculated plant shoots (+/- metal treatment) and shoots from plants treated with *B. coagulans* DUS-M6 siderophore (+/- metal treatment). The greener colouring denotes the lower values (based on calculated relative mRNA levels standardised against the *actin*-2 house-keeping gene) for the specific gene (row) with the transition from green to yellow to red indicating increases in relative mRNA levels (where dark red denotes the highest relative mRNA levels).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative mRNA levels in shoots of uninoculated plants</th>
<th>Relative mRNA levels in shoots of plants inoculated with <em>B. coagulans</em> DUS-M6 siderophore</th>
<th>Relative mRNA levels in shoots of plants treated with <em>B. coagulans</em> DUS-M6 siderophore</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No metals</td>
<td>Fe</td>
<td>Zn</td>
</tr>
<tr>
<td>NRAMP1</td>
<td>1.6034</td>
<td>1.613</td>
<td>1.658</td>
</tr>
<tr>
<td>NRAMP2</td>
<td>0.969</td>
<td>0.947</td>
<td>0.624</td>
</tr>
<tr>
<td>NRAMP3</td>
<td>1.5644</td>
<td>1.618</td>
<td>1.641</td>
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<tr>
<td>NRAMP4</td>
<td>0.9934</td>
<td>0.955</td>
<td>0.949</td>
</tr>
<tr>
<td>NRAMP5</td>
<td>1.2921</td>
<td>1.564</td>
<td>1.509</td>
</tr>
<tr>
<td>NRAMP6</td>
<td>0.9179</td>
<td>1.02</td>
<td>0.964</td>
</tr>
<tr>
<td>CT1</td>
<td>0.7814</td>
<td>0.923</td>
<td>0.8</td>
</tr>
<tr>
<td>CT2</td>
<td>1.0191</td>
<td>1.21</td>
<td>1.092</td>
</tr>
<tr>
<td>ZIP1</td>
<td>1.1087</td>
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<td>1.126</td>
</tr>
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<td>EIN2</td>
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<td>0.869</td>
</tr>
<tr>
<td>SRT1</td>
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<td>0.866</td>
<td>0.881</td>
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<tr>
<td>YSL1</td>
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<td>1.68</td>
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<tr>
<td>YSL2</td>
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<td>2.182</td>
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<td>YSL3</td>
<td>0.5737</td>
<td>0.575</td>
<td>0.585</td>
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<td>YSL5</td>
<td>2.3292</td>
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<td>2.591</td>
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<td>YSL6</td>
<td>1.7818</td>
<td>0.111</td>
<td>7.379</td>
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<tr>
<td>YSL7</td>
<td>2.2243</td>
<td>4.596</td>
<td>2.583</td>
</tr>
</tbody>
</table>
4.0 Discussion

4.1 Identification of the *B. napus* growth promoting bacterial isolate *Bacillus coagulans* DUS-M6

Plant growth promoting bacteria (PGPB) can enhance plant growth through a number of different mechanisms. These include: production of auxins such as IAA; production of enzymes such as ACC deaminase; and increased bioavailability of nutrients such as phosphate or iron through their production of siderophores (Sánchez-Cañizares et al., 2017; Gamalero and Glick, 2011). The study presented here identifies a species of bacteria, *Bacillus coagulans* DUS-M6, that was extracted from soil samples collected from Maryborough in Central Victoria, Australia, which is a major canola growing region of Australia’s Southern grain cropping region. The *B. coagulans* DUS-M6 isolate was identified as a potential PGPB for canola based on its ability to solubilise iron and phosphate, produce IAA and ACC deaminase (Table 2); these are previously established common traits for most PGPBs (Sánchez-Cañizares et al., 2017; Gamalero and Glick, 2011). The isolate was further identified to species using 16S rRNA sequencing. The validation that *B. coagulans* DUS-M6 enhances *B. napus* growth was achieved by inoculating it on plants, at the point of seed planting and observing an increase in plant growth compared to an uninoculated control, over a 2-week growth period (Fig. 5A and B). The use of *Bacillus* and *Paenibacillus* as plant growth promoting bacteria is well reported as they form stress tolerant endospores which can survive under adverse conditions and provide bioformulation solutions (Kloepper et al. 2004; Emmert and Handelsman 1999).

Previous studies have proposed *Bacillus* sp. as suitable candidates for further development and use as commercial PGPB inoculants (Gutiérrez-Mañero, et al., 2001; Zheng et al., 2013; Lugtenberg and Kamilova, 2009; Kumar et al., 2011; Kumar et al., 2012). The most reported *Bacillus* strains shown to have PGPB activities are *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium*, *B. pumilus* and *B. licheniformis* (Çakmakçi et al., 2007; Joseph et al., 2012). *Bacillus amyloliquefaciens* KPS46 increased plant biomass and overall yield (by 24-30%) compared to uninoculated control plants (Buensanteai et al., 2008). Inoculation of *B. subtilis* ALB629 on seeds of cacao plants significantly enhanced root growth (Falcão et al., 2014). Similarly, *B. megaterium* mj1212 increased the root and shoot length of mustard plants (Kang et. al., 2014). It has been also documented that seeds which are inoculated with different selected strains of *Bacillus* can establish themselves in the rhizosphere of different wheat varieties (Juhnke et al. 1987 and Milus and Rothrock 1993).
Four strains of *B. subtilis* currently available as commercial products (Kodiak, Companion, Subtilex and Taegro) have been characterised as reducing pathogens including *Rhizoctonia, Phytophthora, Pythium and Fusarium* on various cropped plants (Table 7 and 8) (Gardener & Fravel 2002). The success of these *B. subtilis* strains across multiple cropped plants and reproducible results in different cropping systems (soil types, climates and irrigation strategies) has enabled their development as commercially available biological control agents (BCAs); and are typically incorporated into the bioformulation of on-seed treatments (coatings) with polymers desiccants, fungicides and key trace metals (Minaxi *et. al.*, 2012; McSpadden Gardener and Fravel 2002) (Table 7 and 8). These on-seed coatings containing *Bacillus* strains have been shown to improve the germination of cowpea; with the modes of action for the enhancement the cowpea germination and growth by the *Bacillus* strains being P solubilisation, antifungal properties and ACC deaminase activity (Minaxi *et. al.*, 2012). Whilst previous studies have identified potential candidates for development as commercial inoculants for canola (Glick 2012; Souza, Ambrosini & Passaglia 2015), they have not yet been developed into commercial products, nor have any of these products been developed specifically for Australian canola cropping systems. Bacterial inoculants must be tailored to specific cropping conditions to ensure optimum performance. *Bacillus coagulans* DUS-M6 offers a potential inoculant that could be used in canola cropping systems in Australia. Based on the data presented in this study, *B. coagulans* DUS-M6 is able to produce IAA, ACC deaminase, solubilise iron and phosphate and enhance canola seedling growth Thus, *B. coagulans* DUS-M6 has the potential to be further developed as a commercial PGPB inoculant for *B. napus* L (canola), which could potentially be incorporated as part of current seed coating formulas.
Table 7: Commercially available *Bacillus* BCAs used in bioformulations for inoculation on various cropped plants (McSpadden Gardener and Fravel 2002; Minaxi *et. al.*, 2012).

<table>
<thead>
<tr>
<th>Commercial name</th>
<th><em>Bacillus</em> strain</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Companion</td>
<td><em>B. subtilis</em> GB03</td>
<td>Control diseases caused by <em>Rhizoctonia</em>, <em>Phytophthora</em>, <em>Pythium</em> and <em>Fusarium</em></td>
</tr>
<tr>
<td>Subtilex</td>
<td><em>B. subtilis</em> MBI600</td>
<td>Control root seed borne diseases of vegetable crops such as soybean, vines, strawberries, cucumber and cereals.</td>
</tr>
<tr>
<td>Serenade</td>
<td><em>B. subtilis</em> QST713</td>
<td>Control downy mildew, early and late blight diseases, <em>Cercospora</em> leaf spot</td>
</tr>
</tbody>
</table>
Table 8: Beneficial *Bacillus* strains used for commercial biological seed treatment

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Target</th>
<th>Crop</th>
<th>Seed treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td><em>Rhizoctonia, Fusarium spp.</em></td>
<td>Cotton</td>
<td>Proprietary formulation</td>
<td>(Kenney 1997), Bayer (2016a)</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em></td>
<td><em>Rhizoctonia solani</em></td>
<td>Soybean</td>
<td>Bacterial suspension</td>
<td>Correa et al, 2009</td>
</tr>
<tr>
<td><em>B. subtilis, Gliocladium catenulatum</em></td>
<td><em>Plasmodiophora brassicae</em></td>
<td>Canola, crucifer vegetables</td>
<td>Bacterial suspension</td>
<td>(Peng G 2011)</td>
</tr>
</tbody>
</table>
4.2 Production of a pyoverdine-like siderophore *Bacillus coagulans* DUS-M6 enhances *B. napus* growth

In order to determine if *B. coagulans* DUS-M6 could be developed into a commercial inoculant, the potential mode of action(s) as to see how this bacterial isolate could be enhancing canola growth was further explored. One of the interesting modes of action predicted for enhancement of *B. napus* L. growth when inoculated with *B. coagulans* DUS-M6 was based on the production of nutrient (Fe) solubilising peptides (siderophores) (Table 2). Siderophores are small peptide-like molecules with specific functional groups which have high affinity ligands attached for ferric ions. They are classified into three main categories based on their structure, type of ligands and functional groups, specifically, carboxylates, hydroxamates and catecholates (Crowley, 2006). Hydroxamate siderophores have stability constant equivalent to 1:1 metal-EDTA complexes with Fe (III) which is proximately equals to that of Fe (III)-EDTA complex (10³⁰), whereas carboxylates and catecholates siderophores have complexes which have stability constants near to Fe (III)- ethylenediamine- N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) (10⁴⁰) (Robert 1992).

Siderophores have been shown to enhance plant growth through increasing environmental trace metal levels. Metals including Fe, Co, Zn, Cu and Mn are essential for optimum plant growth. Bacterially produced siderophores have been shown to chelate these elements from the soil and make them more available to not only the bacteria but also to plants. It is known that siderophores are produced in response not only to limited conditions of iron, they have the ability to interact other metals as well (Evers et al, 1989; Martell et al., 1995; Hernlem et al., 1996). The coordination chemistry involved in interaction of siderophores with metals other than iron has been determined (Boukhalfa et. al.,2002) (Cortese et. al.,2002). Siderophores produced by *Pseudomonas* sp. have the ability to form complex with other metals and this has gained significant attention (Madigan, 2012). The pseudomonad siderophores pyochelin and pyoverdine form complexes with over 15 different transition metal and main group metal ions (Braud et. al. 2009).

Pyoverdines are a type of siderophore that are well documented as chelating Fe and other metals, with purified pyoverdine produced by *P. fluorescens* being commercially available (P8124-Sigma)(Biological activity of secondary metabolites produced by a strain of *Pseudomonas fluorescens*. Boruah HP and Kumar BS Folia Microbiologica 47(4)) Screening of the unknown *B. coagulans* DUS-M6 peptides in growth media showed that it produced a
pyoverdine-like peptide that shared a strong similarity with the commercially available pyoverdine purified from *P. fluorescens* and the siderophores by *Pseudomonas* species have been well-documented to enhance plant growth (Kloepper et. al., 1980; Gamalero and Glick, 2011). Inoculation of barley, wheat, 4 different varieties of maize, cucumber, spinach and corn with the high-pyoverdine producing *P. aeruginosa* 7NSK$_2$ strain resulted in increased plant yields of 10%-24% in comparison to a negative (uninoculated control) and a *P. aeruginosa* 7NSK$_2$ siderophore deficient strain (Hofte et. al., 1991).

Using HPLC to screen production of peptides by *B. coagulans* DUS-M6 that may have a positive influence on plant growth, a pyoverdine-like siderophore was identified. Siderophores such as pyoverdine and pyochelin have previously been shown to chelate up to 16 different types of metals including: Fe$^{2+}$, Fe$^{3+}$, Al$^{3+}$, Ag$^+$, Cd$^{2+}$, Co$^{2+}$, Cr$^{2+}$, Cu$^{2+}$, Ga$^{3+}$, Hg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, Sn$^{2+}$ and Zn$^{2+}$, with the capacity and strength of binding varying for each element and its speciation (Braud et. al., 2009a, b). The order for co-ordination and binding preference for siderophores is typically Fe$^{3+}$ > Fe$^{2+}$ > Cu$^{2+}$ > Ga$^{3+}$ > Mn$^{2+}$ > Ni$^{2+}$ > Zn$^{2+}$, although this can be influenced by the overall chemical conditions of the solution (pH, temperature and presence of other metals) and is largely based on binding kinetics in iron-limited succinate medium (Braud et. al., 2009b). (Teitzel et. al., 2006).

To determine if the pyoverdine-like peptide produced by *B. coagulans* DUS-M6 was able to alter metal availability for *B. napus*, plants were screened in environments with limited metals (no added metals), added Fe, added Zn, added Co, added Cu and added Mn. Under the different metal treatments, the inoculation of plants with *B. coagulans* DUS-M6 was compared against an uninoculated control, the commercially available pyoverdine, as well as the purified pyoverdine-like peptide produced by *B. coagulans* DUS-M6. The effect of the inoculation of *B. coagulans DUS-M6* on enhancing growth, showed that: plants inoculated with *B.coagulans* DUS-M6, plants treated with purified siderophores produced by *B. coagulans* and plants treated with the commercial pyoverdine peptide enhanced plant growth compared to uninoculated control. For all treatment groups (± Fe, Zn, Cu, Co or Mn) the addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased root and shoot biomass by 2-6 mg in comparison with their respective uninoculated controls. This showed that not only was *B. napus* growth was enhanced under the different metal treatments by inoculating with *B. coagulans* DUS-M6 cells, but the pyoverdine-like peptide produced by *B. coagulans* DUS-M6 was also capable of enhancing
plant growth in the absence of the bacteria – most likely due to increasing levels of these essential metals in the plants. This data confirms that PGPB can enhance plant growth through production of peptides that chelate and solubilise metals in the environment. Moreover, it shows that the purified peptides themselves, such as the pyoverdine-like siderophores produced by *B. coagulans* DUS-M6, can be just as effective as the PGPB itself at enhancing plant growth (Kloepper *et. al.*, 1980; Gamalero and Glick, 2011; Hofte *et.al.*, 1991) (Siebner-Freibach *et. al.*, 2003: Verma *et. al.*, 2011).

4.3 Effect of *B. coagulans* DUS-M6 cells and the pyoverdine-like siderophore produced by *Bacillus coagulans* DUS-M6 on uptake of essential metals in *B. napus*

The ability for bacterially produced siderophores to assist in plants meeting their required iron quota for growth have been characterised for a range of plant-microbe associations including: radiolabelled iron ($^{59}$Fe) uptake studies showing the internalisation of Fe associated with the siderophore pyoverdine produced by *Pseudomonas fluorescenes* C7 from solution into tissue of *Arabidopsis thaliana* (Gérard Vansuyt 2007); *Chryseobacterium spp*. C138, isolated from *Oryza sativa* showing Fe mobilisation to the roots of iron-deficient tomato plants (Radzki *et al.* 2013) ferrioxamines (a type of siderophore) produced by *Azadirachta indica* shown to increase Fe levels in plants leading to increased shoot and root growth (Siebner-Freibach *et. al.*, 2003: Verma *et. al.*, 2011); and inoculation of *Sedum plumbizincicola* with *Bacillus* spp. SC2b increased the biomass and further increased the accumulation of Zn and Cd in the shoots and roots of the plant (Ma *et al.* 2015)

Using ICP-MS, the effect of *B. coagulans* DUS-M6 cells and the pyoverdine-like siderophore produced by *Bacillus coagulans* DUS-M6 on the uptake of the essential metals in *B. napus* roots and shoots Fe, Co, Cu, Zn and Mn was measured. The levels of Zn, Co and Mn were significantly higher in inoculated plants roots, compared with uninoculated plants roots (Table 3). Inoculation of plants with *B. coagulans* DUS-M6 significantly increased levels of Zn and Mn in plants roots beyond control levels for all other treatments (Table 3). In plant roots, the addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe, Co, Cu and Mn accumulation in comparison to controls (Table 3). The addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe, Co and Cu levels above all other treatments (Table 3). The addition of the commercial pyoverdine increased Fe and Co uptake significantly compared to control
plants, respectively, but had less effects overall compared to the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 (Table 3).

The accumulated levels of Fe, Zn and Cu were significantly higher in inoculated plants shoots, compared with uninoculated plants shoots (Table 4). In plant shoots, the addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe, Zn, Cu and Mn accumulation in comparison to controls (Table 4). The addition of the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly increased Fe and Mn uptake in plant shoots above levels of all other treatment groups (Table 4) and the commercial pyoverdine significantly increased Fe and Zn levels in plant shoots (Table 4). Significantly, for Co, the levels taken-up into plant shoots were significantly lower for inoculated plants or when the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 or the commercial pyoverdine was added, with Co levels reduced by 0.5, 1.5 and 0.5 nM per mg of dry tissue respectively, with the purified siderophore-like peptide produced by *B. coagulans* DUS-M6 significantly reducing Co levels in comparison to all other treatment groups (Table 4). Overall, we have shown for the first time that *B. napus* plant growth increased as result of the uptake of essential trace elements in plants inoculated with either the bacteria themselves or treated with the purified pyoverdine-like siderophore.

4.4 Effect of *B. coagulans* DUS-M6 cells and the pyoverdine-like siderophore produced by *Bacillus coagulans* DUS-M6 on metal uptake and homeostasis systems in *B. napus*

The sensing, uptake, storage and efflux of trace elements are essential for maintaining their homeostatic levels at the cellular level. Homeostatic levels of trace elements are maintained through the tight regulation of systems involved in metal uptake, translocation of metals within and between cells and reducing free intracellular levels by efflux (Yanan et. al. 2018). If levels of trace elements become too low or too high, cellular metabolism and overall plant growth become reduced (Yanan et al. 2018). Trace elements such as Fe, Co, Cu, Mn and Zn are all essential as cofactors for enzymatic activity, with plants in particular having a higher requirement over other organisms due to their requirement of metal cofactors for the photosystem and antioxidant systems that breakdown the by-products of oxygen evolving photosynthesis such as reactive oxygen species (ROS) (Yanan et. al. 2018). Compounding the pressures on plants to meet their metal quota for optimum growth, is the restricted abundance and availability of trace elements in many soils, particularly those of agricultural
systems. Trace element (or transition metal) levels are relatively limited in many soils and their availability is further restricted by interaction of these metals with other soil constituents (Yanan et al. 2018). The depletion of metals from soils by repeated cropping, further limits plants ability to meet their quota for optimum growth. This is where the assistance of nutrient scavenging by bacteria and their products may assist plants to gain access to these essential metals – facilitating plant growth.

Searches of the B. napus genome in the National Centre for Biotechnology Information (NCBI) database revealed the presence of the following putative metal transporters: six putative natural resistance-associated macrophage protein (NRAMP) encoding genes (NRAMP1, NRAMP2, NRAMP3, NRAMP4, NRAMP5, NRAMP6), the putative copper transporters CT1 and CT2, a putative Zrt Irt-like transporter (ZIP2), seven yellow stripe-like genes (YSL1, YSL2, YSL3, YSL5, YSL6, YSL7 and YSL8) and the putative SRT1 and EIN2 transporters, which shared low similarity with the Zrt Irt-like transporter family. Using qRT-PCR, expression of putative genes involved in metal sensing uptake and homeostasis in roots and shoots of B. napus plants grown under different metal treatments (no added metals, ± Fe, ± Zn, ± Co, ± Cu or ± Mn) and either inoculated with B. coagulans DUS-M6 cells or treated with the pyoverdine-like siderophore were measured to see if changes in gene expression correlated to increased delivery of metals into plants by the bacteria and/or the siderophores.

Generally, the different metal treatments combined with the addition of either B. coagulans DUS-M6 cells or the pyoverdine-like siderophore resulted in changes in relative mRNA levels across all genes, indicating that the treatments were affecting metal levels in the plants, (as substantiated by the metal uptake experiments) and altered the expression of the metal sensing, uptake, storage and efflux systems. These findings provide some insights at the molecular level that the addition of the bacteria or their purified siderophore facilitated the enhancement of plant growth by increasing the uptake of trace elements in the canola plants. Characterisation as to which of the target genes investigated here are central to the PGPB effects observed in this study requires a more detailed investigation of the individual gene changes over a shorter and more scrutinising temporal scale. The further addition of information gained from experiments that localise and track the movement of metals from environment (soil medium) into plants (uninoculated, inoculated or treated with purified siderophores) using radiolabelled uptake studies will enable correlation of the gene expression against metal uptake and mobilisation within tissue and cells.
4.5 Conclusion

This study shows for the first time that a *Bacillus* spp. can be isolated as a suitable PGPB for use on canola. Moreover, the pyoverdine-like siderophore produced by *B. coagulans* DUS-M6 was shown to mobilize metal ions such as Fe, Zn, Cu, Co and Mn, increasing their availability and uptake into plants – resulting in enhanced plant growth. Whilst efforts have been made to better understand the ecology and management of numerous PGPB, their development as commercial products remain a challenge. Currently, there are no commercially available *Bacillus* based inoculants for use with canola – largely due to incomplete characterisations, undetermined modes of action and incompatibility across cropped plants and cropping systems. The information gathered thus far for *B. coagulans* DUS-M6 shows it has potential for further development as a commercial PGPB for canola; however, greater understanding of its ability to perform in a field setting, by using it as an inoculant in multiple field trials is required. The characterisation of the pyoverdine-like siderophore produced by *B. coagulans* DUS-M6 and testing of its effectiveness as a natural metal-chelate could lead to its development as a commercial product for enhancing nutrient levels in plants – providing a novel organic alternative to delivering nutrients to cropped plants.
4.6 Outcomes

Screening bacteria isolated from soil samples for known PGPB traits, their effect on canola growth and 16S rRNA sequencing lead to the identification of Bacillus coagulans DUS-M6. Using HPLC bacterial culture media was analysed to determine if there were any obvious potential peptides or metabolites produced by B. coagulans DUS-M6 that may be attributed to enhancing the growth of canola. The production of a siderophore-like peptide with high similarity to a pyoverdine standard was determined.

Based on the function of siderophores such as pyoverdine to alter the bioavailability of metals in soils, the effect of the purified pyoverdine-like siderophore produced by B. coagulans DUS-M6 was further screened for its ability to increase metal levels in plants as well as plant growth, which it was shown to significantly alter metal levels in plant and their growth.

Using qRT-PCR, the alterations in expression of genes that encode for products that are involved in the sensing, uptake, storage and efflux of metals in B. napus (uninoculated, inoculated or treated with the purified siderophore) were measured; with the different metal treatments combined with the addition of either B. coagulans DUS-M6 cells or the pyoverdine-like siderophore resulting in changes in relative mRNA levels across all genes.

This study identifies a new PGPB isolate B. coagulans DUS-M6 for use on canola and shows that the pyoverdine-like siderophore produced by B. coagulans DUS-M6 mobilizes the metal ions Fe, Zn, Cu, Co and Mn in soil, increasing their availability and uptake into plants – resulting in enhanced plant growth.
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