Tracking beneficial *Streptomyces* strains in plant shoot, roots and rhizosphere soils

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Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma, and to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Lovepreet Singh

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Abbreviations

µl, ml	microlitre, millilitre
µM, mM	micromolar, millimolar
µg, mg, g	microgram, milligram, gram
%	percent
bp	base pair
DNA	Deoxyribonucleic acid
min	Minute (s)
rpm	Rotation per minute
MQ water	Milli Q water
˚C	Degree Celsius
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase chain reaction
PGP	Plant growth-promoting
BGC	Biosynthetic gene cluster
sp.	Species
IAA	Indole Acetic Acid
ABARES	Australian Bureau of Agricultural and Resource Economics
GRDC	Grain research and development corporation

Abstract

Bacteria are ubiquitous in the environment. Among these bacteria, *Streptomyces* which are an important genus of Actinobacteria can be found in the soil and produce a number of bioactive secondary metabolites. *Streptomyces* can also be found in plants as endophytes to promote the plant growth and to suppress plant pathogens. Endophytic population also assist in the plant growth by alleviating biotic stresses (disease control) and abiotic stresses such as drought. These endophytes can promote plant growth and yield, suppress pathogens, may help to remove contaminants, solubilize phosphate, or contribute in nutrient acquisition in plants. These treatments were conducted on plants using whole live cells of *Streptomyces*. Wheat and chickpea plants grown under application of *Streptomyces* were selected to monitor the presence of bacterial strain. To monitor the presence of these endophytes some techniques needed to be developed. There are many different methods to isolate and characterise bacterial strains, but it is difficult to track a specific strain that was added to a plant. These pitfalls can be avoided by using whole genome next generation sequencing and target conserved genes with high single nucleotide polymorphism. Primer designing on the highly polymorphic region could be helpful to identify and track specific *Streptomyces* strain in plant and soil samples. To ensure the primer specificity, optimization of strain specific primers was first performed by using *in silico* bioinformatic tools. Conventional PCR was used to confirm the specificity of designed primers for selected strains versus other closely related *Streptomyces* strains. Then selected *Streptomyces* strains were detected and quantified in plant shoots and roots and soil samples using quantitative PCR. *Streptomyces* strains in different plant and soil samples were detected in seeds and plants roots. The final DNA concentration of specific strains was calculated using a standard curve obtained following serial dilution of the DNA of pure cultures. The presence and quantification of *Streptomyces* strains will be helpful to optimize the concentration of inoculant.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Overview

Actinobacteria is an essential phylum of bacteria that enhance the growth of plants by producing a number of compounds including secondary metabolites, phytohormones (Coombs et al. 2004a; Franco et al. 2007; Solans et al. 2011). These biologically active compounds exhibit a remarkable array against pathogens with their antifungal, antimicrobial, antiparasitic, immunosuppressive and antitumor activities (Franco et al. 2007; Schrey et al. 2008). Actinobacteria can be found in and on any plant part, but the majority can be found in the roots (Araujo et al. 2017; Bulgarelli et al. 2012). Rhizosphere-associated soil contains a large actinobacterial population as compared to non-rhizosphere associated soils (Intra et al. 2011). Chemicals produced by the roots of plant is the reason for the large microbial population present in the rhizosphere (Mcnear Jr. 2013). *Streptomyces* is the largest genus of Actinobacteria. *Streptomyces* is considered as an important biological entity in the soil as they produce various antibiotics to suppress fungi and pathogens (Ningthoujam et al. 2009). Monitoring the presence of *Streptomyces* in the rhizosphere and plant could be helpful to understand the interaction of the bacterial population with the host plant.

1.2 Plant diseases and yield loss

Crop pathogens are the primary concern that affects the crop industry in Australia. The effect of the pathogen on crops increases the probability to manifold for less plant growth and reduction in yield (Misk et al. 2011). Along with other plant diseases, root rot is considered as the most common pathogen attack on crops. Root rot affects the yield of key crops, i.e. wheat and barley, in most of the countries in the world. On average, a 34% yield reduction can be expected in cereals affected by root rots, especially in Australia with losses over 26%; countries reporting losses include Brazil 15–38%, Canada 5–28%, France 15–75%, Italy, Morocco 4–6%, Turkey, and USA 40–50% (Orakci et al. 2010). Australia contributes significantly to wheat world production (Law et al. 2017) and global wheat trade (15%). *Rhizoctonia* and *Pythium*

are considered as the most common pathogens that lead to a loss of $77 million in southern Australia (GRDC) (https://grdc.com.au). According to ABARES (http://www.agriculture.gov.au/abares) survey (2017), the demand for wheat increased by 6% as compared to last year.

1.3 Pathogens control

Some conventional techniques can be used to control the growth of pathogens, including crop rotation, alternative cropping system and by soil disturbance (Guetsky et al. 2002). These methods are not very useful because pathogens can survive on a broad range of host and it can survive for a longer time in soil (Liu et al. 2011). Chemical control is another alternative, but it leads to environmental pollution and is not desirable for the long term. In the last 40 years, the use of synthetic nitrogen has increased seven times and the use of pesticides has increased three times. Application of chemical control will increase three times more by 2050 if current cultivation practices are maintained (Fox et al. 2007).

1.3.1 Endophytic Biocontrol

In the evolutionary process, habitat of plants moved from oceans to land and faced so many problems to get fit into the atmosphere of low soil nutrients and high carbon dioxide. Under those conditions, fungi improved its interactions with plants and helped them to be more adapted to the terrestrial conditions (Bonfante et al. 2010). Bacteria and fungi accumulated genetic variations to adapt to the plant microenvironment (Germaine et al. 2004). These microorganisms were later called endophytes. Endophytes are the microorganisms that can be isolated from the surface-sterilized plant parts and they do not cause any harmful effects to the plant (Arora et al. 2017). Bacterial endophytes have multiple applications in pharmaceutical and other industrial sectors (Figure 1), i.e. medical and antibiotics industries, pollution control and phytoremediation, plant disease control, yield and growth promotion. Plant diseases can

be controlled with biocontrol agents to increase the quality crop yield without any chemical control for environment safety (Pal et al. 2006). Bacterial endophytes are considered as microbial populations that inhabit inside the host plant for their lifespan or part of life cycle without causing any harmful effect to host (Hardoim et al. 2008). Endophytic bacteria can produce phytohormones, antibiotics that enhances the plant ability against biotic and abiotic stresses (Gaiero et al. 2013). Plants without interactions with endophytes have less ability to fight with pathogens and environmental stress (Timmusk et al. 2011). This bacterial population can produce novel secondary metabolites used in agriculture (Santoyo et al. 2016). In recent research, *Bacillus megaterium* and *Bacillus subtilis* were extracted from wheat seed as endophytic bacteria and showed the resistance to *Fusarium* head blight (FHB) (Pan et al. 2015). Endophytic bacteria interact with plants and respond through many mechanisms to show a beneficial effect.

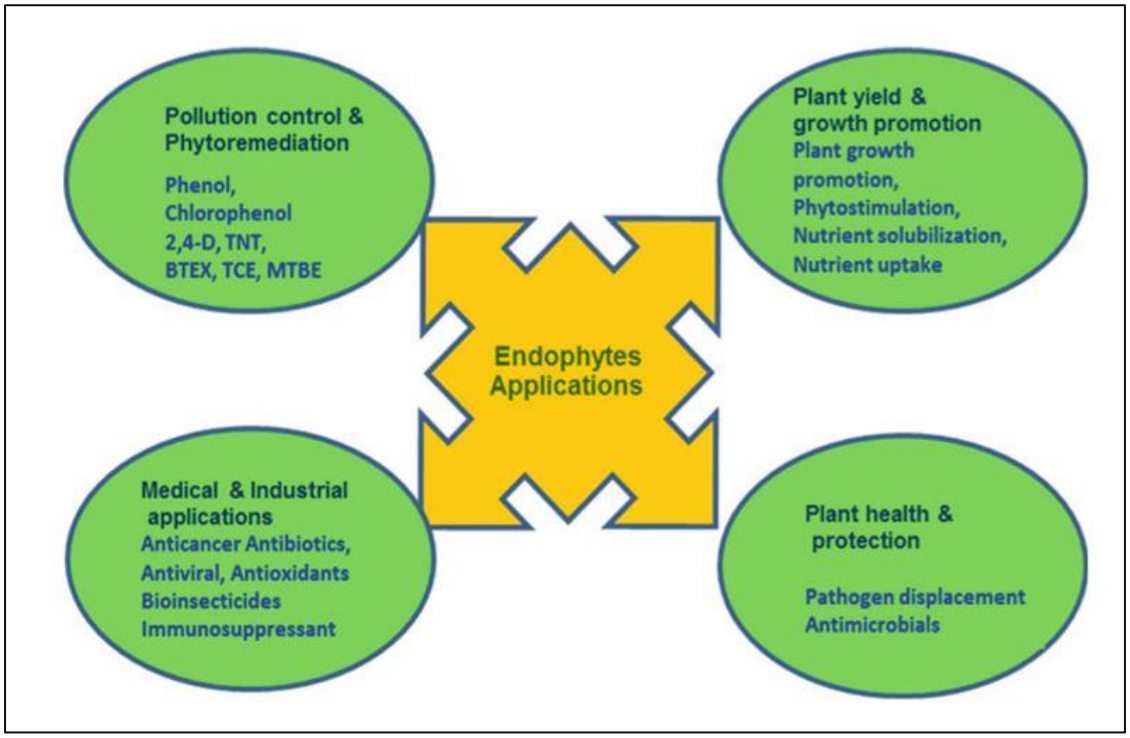


Figure 1: Applications of endophytes in different sectors, i.e. pollution control, plant growth, medical applications and plant health(K. Maheshwari. 2017)

1.3.2 Actinobacteria as biocontrol agents

Actinobacteria are gram-positive bacteria with a positive impact on the root zone in plants. Actinobacteria represent a large number of taxonomic groups in the domain bacteria (Singh et al. 2018). Chemical control of pathogens in crops and legumes leads to many health fastidious diseases (Gerhardson. 2002). Use of fungicides and pesticides of crops is not always cost-effective for farmers (Misk et al. 2011). Chemical control of pathogens pollutes the soil and environment. Biocontrol of pathogens using harmless bacteria could be a promising solution (Singh et al. 2018). Biocontrol agents reduce the rate of pathogen colonisation by following multiple pathways or mechanisms (Table 1). Endophytic Actinobacteria produce many bioactive products such as antibiotics, secondary metabolites, enzymes, antitumor compounds, which makes it most suitable for biocontrol agent against many diseases (Barka et al. 2016). These bacteria also stimulate the plant growth by nitrogen fixation, phosphorus uptake and restriction of pathogens by disrupting cell wall, nutrient competition.

Table 1: Action of mechanisms for biocontrol agents (Pal et al. 2006).

Type	Mechanism	Examples
Direct antagonism	Hyper parasitism/predation	Lytic/some nonlytic mycoviruses
		Ampelomyces quisqualis
		Lysobacter enzymogenes
		Pasteuria penetrans
		Trichoderma virens
Mixed-path antagonism	Antibiotics	2,4-diacetylphloroglucinol
		Phenazines
		Cyclic lipopeptides
	Lytic enzymes	Chitinases
		Glucanases
		Proteases
	Unregulated waste products	Ammonia
		Carbon dioxide
		Hydrogen cyanide

	Physical/chemical interference	Blockage of soil pores Germination signals consumption Molecular crosstalk confused
Indirect antagonism	Competition	Exudates/leachates consumption Siderophore scavenging Physical niche occupation
	Induction of host resistance	Contact with fungal cell walls Detection of pathogen-associated, molecular patterns Phytohormone-mediated induction

In agriculture, *Streptomyces* play a significant role as they produce various antifungal and antibacterial metabolites along with several plant growth-promoting (PGP) traits (Table 2). *Streptomyces* can produce relevant and novel antibiotics when living as endophytic bacteria. *Streptomyces* are helpful in lysis of fungal hyphae by producing chitinases and glucanases (Taechowisan et al. 2009). *Streptomyces* can reduce the growth of pathogens by disrupting the cell wall of the pathogen (Chamberlain et al. 1999). These bacteria also showed positive results in the reduction of mycelium growth by producing volatile metabolites (Nourozian et al. 2006). When compared to other endophytic genera, *Streptomyces* are considered to be most useful to control pathogens in a different field and glasshouse trials for wheat (Franco et al. 2007) and legumes (Misk et al. 2011). Results from different researchers unveil the dominance of *Streptomyces* in wheat and legume plants (Coombs et al. 2003), rice (Tian et al. 2007), banana (Cao et al. 2005) and medicinal plants (Khamna et al. 2009a).

Table 2: List of Streptomyces isolated from plants or the rhizosphere showing plant growth-promoting (PGP) activity (Vurukonda et al. 2018).

Species	Host	PGP Traits/Observed Effects in Plants
Streptomyces sp.	Rice, chickpea	Nutrient uptake and plant growth-promoting
Streptomyces sp.	Mung bean	Plant growth enhancement
Streptomyces sp.	Soybean	Nutrient uptake and plant growth-promoting
Streptomyces atrovirens, *S. griseoviridis*, *S. lydicus*, *S. olivaceoviridis*, *S. rimosus*, *S. rochei*, *S. viridis*	Rhizosphere of different plants	Auxin/IAA production
Streptomyces sp.	Rhizosphere of different plants	Gibberellin biosynthesis
Streptomyces sp.	Soil	Synthesis of IAA and siderophore production
Streptomyces rochei, *S. carpinensis*, *S. thermolilacinus*	Wheat rhizosphere	Production of siderophore, IAA synthesis, and phosphate solubilization
Streptomyces sp.	Soil	Siderophore production, phosphate solubilization, and N_2 fixation
Streptomyces olivaceoviridis, *S. rochei*	Wheat	Auxin, gibberellin, and cytokinin synthesis
Streptomyces sp.	Rice	Stover yield, grain yield, total dry matter, and root biomass increase
Streptomyces sp.	Soil	β-1,3-Glucanase, IAA, and HCN synthesis

Note: IAA: Indole-3-acetic acid; HCN: Hydrogen cyanide.

1.4 Commercial application

As a commercial application, *Streptomyces* as biocontrol agents can be used in many ways like seed treatment, foliar spray, soil inoculation (Gopalakrishnan et al. 2014; Law et al. 2017). Many commercial products derived from *Streptomyces* are available in the market for agriculture purpose. Blasticidin-S derived from *S. griseochromogenes* against rice blast is available in the United States of America. Streptomycin (Agrimycin, Paushak, Cuprimicin 17, AAstrepto 17, AS-50, Dustret, Cuprimic 100) produced using *Streptomyces griseus* helps to control various bacterial diseases like root rot, *Xanthomonas oryzae, Xanthomonas citri* in the different crop, vegetable and fruit plants (Hamedi et al. 2015; Saxena. 2014).

Table 3: List of Streptomyces based products available in the world market (Vurukonda et al. 2018)

Commercial Product Name	Organism as Active Substance	Registered as Microbial Pesticide	Targeted Pest/Pathogen/Disease
ACTi novate, Novozymes BioAg Inc., USA	*S. lydicus WYEC 108*	Canada, USA	Soilborne diseases, viz. *Pythium*, *Fusarium*, *Phytophthora*, *Rhizoctonia*, and *Verticillium*; foliar diseases such as powdery and downy mildew, *Botrytis* and *Alternaria*, *Postia*, *Geotrichum*, and *Sclerotinia*
Mycostop, Verdera Oy, Finland	*Streptomyces K61*	EU, Canada, USA	Damping off caused by *Alternaria* and *R. solani* and *Fusarium*, *Phytophthora*, and *Pythium* wilt and root diseases
Mykocide KIBC Co. Ltd. South Korea	*Streptomyces colombiensis*	South Korea	Powdery mildews, grey mold, brown patch
Bactophil	*Streptomyces albus*	Ukraine	Seed germination diseases
Actin, Sri Biotech Laboratories India Ltd., India	*S. atrovirens*	India	Fungicide

1.5 Secondary metabolites diversity

Secondary metabolites are compounds that helps host in vital functions such as protection, competition, and species interactions, but are not necessary for survival. Microorganisms can produce chemically diverse novel metabolites (Berdy. 2005; Vicente et al. 2018). Actinobacteria produce a high diversity of secondary metabolites. *Streptomyces* spp. produce over half the secondary metabolites among the total bacterial population to improve plant growth worldwide (Doroghazi et al. 2013). In closely related bacterial strains from the same species, despite of similarity in their genomes, these strains can be very diverse in the production of secondary metabolites. The genes coding the production of secondary metabolites located in bacterial genomes can be organized in biosynthetic gene clusters (BGCs) (Fischbach et al. 2008; Osbourn. 2010). The *Streptomyces* genome includes various gene clusters that code for different metabolites. Most of the BGCs encode for essential metabolites through pathways like polyketide synthases (PKS), non-ribosomal synthetases (NRPS), and PKS–NRPS hybrids. Some of the gene clusters shared by the majority of the *Streptomyces* sp. present in soil generally encodes for hopene, geosmin, ectoine, terpenes (Kim et al. 2015; Vicente et al. 2018). Geosmin is responsible for the odour of soil and hopene can provide stability to bacterial membrane at high temperatures and acidity (Kim et al. 2015). Sometimes, it also consists of transportation genes for exportation of the produced SMs and resistance genes that prevent self-destruction in the producers (Tran et al. 2019). Some other gene clusters which are not shared by the majority of *Streptomyces* sp. related to performance of defence mechanisms or carbohydrate transport/metabolism (Kim et al. 2015). This concept of novel genes present in specific bacterial strains led to research towards PAN genome. The PAN genome is the entire gene set of all strains of a species. It includes genes present in all strains (core genome) and genes present only in some strains of a species (variable or accessory genome).

1.5.1 Whole-genome sequencing and antiSMASH

Whole-genome sequencing (WGS) has great potential to resolve complex evolutionary problems. As compared to traditional methods like pulse-field gel electrophoresis, WGS has high discriminatory power to detect closely related strains from the same species (Yang et al. 2019). WGS analyses are conducted using pure bacterial cultures. WGS potentially provides information about isolates, which includes species, strain type, antibiotic resistance, virulence (Besser et al. 2018). Before the emergence of WGS, the total number of BGCs discovered in bacterial population was limited. For example, the number of known BGCs in *Streptomyces coelicolor* A3(2) was four, which changed to 18 after whole-genome sequencing (Bentley et al. 2002). Cost effective WGS is rapidly emerging the field of microbial genomics and mining of secondary metabolites through bioinformatics analyses (Bachmann et al. 2014; Baltz. 2016; Palazzotto et al. 2018). Illumina genome sequencing already presents the effectiveness for genome mining through sequence analysis of single genes, gene clusters or full chromosomes (Castro et al. 2018).

Latest secondary metabolite analytic pipeline Antibiotics & secondary metabolites analyses shell (antiSMASH) could be a beneficial tool for better reflection of bacterial genome metabolite diversity (Blin et al. 2016; Blin et al. 2019; Weber et al. 2015). AntiSMASH is used to predict the all possible biosynthetic gene clusters using the whole genome sequence of a bacterial isolate. AntiSMASH could provide the BGCs diversity in closely related bacterial strain from the same species with high genome similarity. In a nutshell, whole genome sequencing and antiSMASH together could provide the depth in the diversity of gene cluster and novel secondary metabolite production in strains with high genome similarity.

1.6 Next-generation sequencing

In the modern era of technology, next-generation sequencing (NGS) changed the field of genomic research in microbes (Forde et al. 2013). These latest methods of sequencing allow the study and characterisation of genome or genes in more depth to analyse diversity and content in the microbial population (Pérez-Losada et al. 2017). In NGS, an advance methodology has been used, which brought down the cost and sequencing time as compared to Sanger sequencing. NGS could generate an efficient workflow with WGS to get more information about the species, serotype, virulence characteristics and antibiotic resistance (Rossen et al. 2018). NGS could provide high discriminatory power to target biosynthetic genes and their copies in the genome sequence of targeted isolate for strain tracking.

1.7 Bacterial genetic diversity

Bacterial strain typing is a methodology that allows the identification of the multiple strains and characterisation of genetic diversity in the host plants (Yıldırım et al. 2011). Bacterial strain typing can be organised generally by the genotyping method. In genotyping strategies, a specific strain is detected and characterised on the bases of genetic content (Yıldırım et al. 2011). Many different methods can be done for bacterial genotyping, including DNA banding pattern, DNA hybridisation strategies and DNA sequencing (Taha et al. 2004). In DNA banding-based methods, known or unknown regions are digested with restriction enzymes and analysed comparatively. In DNA hybridisation-based methods, probes are used to hybridise and analyse the DNA content. The most accurate method for strain discrimination uses DNA sequencing and specific gene sequences are used to identify the bacterial strains (Versalovic et al. 2002). DNA sequencing can be used by employing next-generation sequencing for characterisation and identification of the specific strain.

In Multilocus sequencing typing (MLST), nucleotide variation of fragments in the sequences is examined. Generally, housekeeping genes are selected to find polymorphism between these genes (Guo et al. 2008; Li et al. 2009; Pérez-Losada et al. 2017). In *Streptomyces*, generally, five protein-coding genes have been used for bacterial strain tying i.e. *atpD, gyrB, recA, rpoB* and *trpB* (Guo et al. 2008). MLST is generally preferred over other classic techniques of strain typing due to three critical advances in the microbiology, i.e. evolution and population biology information of bacteria, database availability of genetic sequence and high throughput sequencing of nucleotides (Li et al. 2009; Maiden. 2006). Sometimes, MLST fails to identify closely related strains from the same species with high genome similarity. To overcome this limitation of strain identification, use of single nucleotide polymorphism (SNP) with whole-genome sequencing could provide more detailed information on the bacterial isolates.

1.7.2 Single nucleotide polymorphisms and bacterial diversity

SNPs have been considered a prime target to define microbial taxonomy and detection of specific bacterial strains (Jung et al. 2012). SNPs & WGS based strain typing is generally performed via reference-based mapping (Bryant et al. 2013; Reuter et al. 2013; Schürch et al. 2018). In high-quality SNP approach, there is a requirement of the closely related reference genome to generate the polymorphism between related isolates (Forde et al. 2013; Nadon et al. 2017). Screening of SNPs from a bacterial genome could be used for sequence analyses or some other practical strategies (Araujo et al. 2015). In closely related strains from the same species, the genes with different nucleotide sequences are present in other strain sequences. Presence of enough polymorphism in those gene sequences leads to the identification of a specific marker in strain.

1.8 Selection of *Streptomyces* strains

Several studies in the Department of Medical Biotechnology (Flinders University, Australia) on endophytic bacteria revealed that actinomycetes play a significant role to promote plant's growth and reduces the pathogen growth in the roots (Coombs et al. 2003). Field trails around Adelaide (South Australia) showed that the wheat seeds coated with *Streptomyces* strain EN27 yield the same as seeds grown under the commercial application fungicide Jockey® (C Franco et al. 2007). Apart from this, seeds treated with *Streptomyces* strain EN27 yields 5-15% more than untreated ones (Franco et al. 2007). In the early stage of root growth, *Streptomyces* was observed to enter the plant through the lateral root junction (Figure 2) and are considered as the most potential entry area for actinomycetes (Araujo et al. 2017; Franco et al. 2007). Along with other bacteria, Actinobacteria and fungi colonise at the mid-growth stage of the plant (Araujo et al. 2017). To understand better the interaction of *Streptomyces* strains with roots and rhizosphere, strain tracking can play a substantial role to identify the specific strain interaction with the plant.

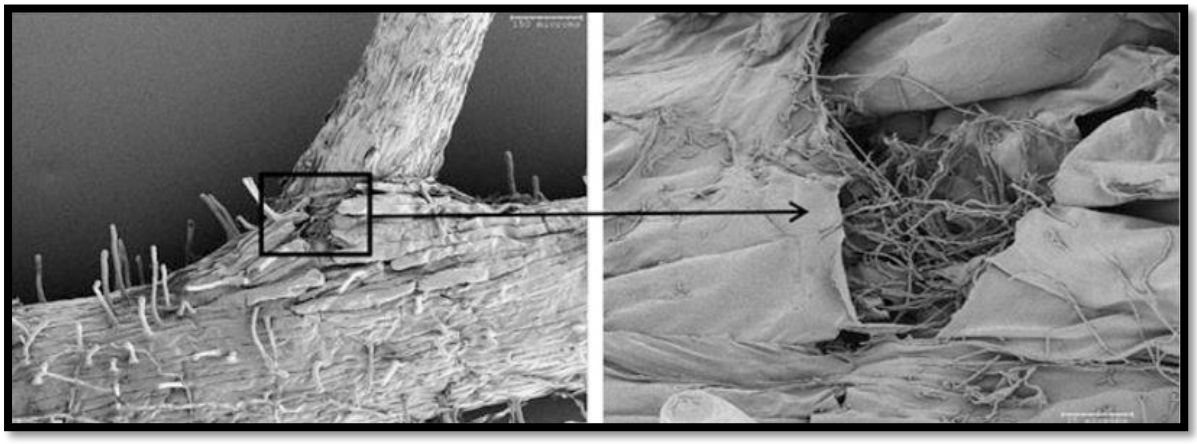


Figure 2: Cryo-SEM micrograph showing an abundance of actinobacteria on lateral root junctions of wheat (Araujo et al. 2017).

In Flinders University, a total of 9 *Streptomyces* strains were isolated from surface sterilised roots of wheat, canola and chickpea. These *Streptomyces* strains EN16, EN23, EN27, HCA1273, BD141 (wheat), CSE94, CSE 46 (canola), CP200B, CP21A2 (chickpea) showed

positive results to control fungus (petri dish assay) and to promote plant growth (glass house trails). After screening, these bacterial isolates were sequenced using the Illumina MiSeq sequencing platform (AGRF, Sydney, Australia) (Franco et al. 2016). Genomic sequence of strain EN16, EN23 and EN27 (C Franco et al. 2016); HCA1273 and BD141; CP200B and CP21A2; CSE94 and CSE46 are closer to each other respectively. In wheat, these isolated *Streptomyces* strains have already displayed the potential to control fungus growth and increase in yield (Franco et al. 2007). In the Department of Medical Biotechnology, CP200B showed positive results to increase the total biomass of chickpea under glasshouse trails (unpublished results). CP200B also displayed the potential in the chickpea plant to maintain green mass under drought conditions. Unique transporter biosynthetic gene sequences and their copies present in closely related strains with different nucleotide sequence could be targeted to design primer and track the specific *Streptomyces* strain in rhizosphere and plant roots.

1.8.1 Strain tracking

In these closely related strains, conserved biosynthetic genes could be used for specific strain tracking as they have the potential to reflect enough polymorphism for marker development. Multiple copies of transporter genes are present in bacterial genome sequence with different nucleotide sequences. Using polymorphic region among transporter biosynthetic genes and their copies in closely related strains, the development of strain-specific primers could be possible for *Streptomyces* strain tracking. The target biosynthetic genes are peptide synthase (*peps*), polyketide synthase (*pks*), non-ribosomal peptides synthases (*nrps*). These gene sequences must be aligned in silico to get the highly polymorphic region. Primer designing on highly polymorphic regions must be conducted based on PCR optimised conditions, i.e. size, structure, compatibility. Nucleotide fragments sized between 80 bp to 220 are useful on the bases of further In vivo experiments, i.e. Amplification, sequencing and quantification of mini fragments for specific strain tracking in the plant roots and other parts.

1.9 AIM

Developing multiple assays for tracking relevant *Streptomyces* strains in plant roots and soil. I targeted several conserved nucleotide sequences to find polymorphism and develop markers. These strain specific markers can be used to track specific *Streptomyces* strains in plant. Such specific markers were tested in silico (bioinformatic tools) then in the lab using conventional PCR for screening of molecular markers. Lastly, selected markers for quantitative PCR were used to track the bacterial strain in the soil and roots at different growth periods.

1.9.1 Hypothesis

Conserved and polymorphic gene sequences can be the ideal target to design primer for specific strain tracking in roots and rhizosphere to study the interaction between *Streptomyces* strain and plant. Strain tracking in the soil and plant roots could reveal the presence and concentration of specific strain at different growth periods.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Using AntiSMASH to identify BGCs

Nine *Streptomyces* strains were selected for the experiment based on their past performance in the different experiments conducted in the Medical Biotechnology Department, Flinders University. To analyse the secondary metabolite diversity, genomes of these *Streptomyces* strains were uploaded to AntiSMASH (https://antismash.secondarymetabolites.org/#!/start) (Blin et al. 2019). The results were retained and organised in Microsoft Excel (Microsoft, USA) for further analyses of biosynthetic gene clusters that produce a wide range of secondary metabolites. AntiSMASH compares the genomic regions of specific strains with the sequences of well-known gene clusters included in the database (Blin et al. 2019; Weber et al. 2015). It reveals the potential biosynthetic compounds produced by the submitted strain along with a probability based on the number of genes present in the target genome and the cluster. Similarity hit of 100 mean a high chance of the strain produce a specific metabolite by including all the necessary genes for the complete pathway (Blin et al. 2016).

2.2 Selection and alignment of housekeeping and transporter biosynthetic genes

In the first part, several gene sequences from the same species and different strains were selected based on their performance in previous research. Along with housekeeping genes i.e. *atpD, gyrB, recA, rpoB* and *trpB,* another sequences of 15 genes (*MethH, tafA, prcA, dnaK, hrdB, sigB, whiG, shbB, rbpA, trxA, trxB, hrdC, lcp)* were compared to get decent polymorphic regions at the strain level. Multiple alignments were performed in each gene to find highly polymorphic and unique nucleotide sequence using software Geneious prime (https://www.geneious.com, Biomatters, Ltd. New Zealand). Finding of a unique polymorphic region in some other conserved genes could provide the nucleotide sequence to design marker in *Streptomyces* strains selected for this experiment. This approach did not give any significant results to target specific polymorphic region in genes present in selected genomes for this experiment.

In the second part, multiple copies of semi-conserved transporter genes found within a single genome were compared. Some antibiotic biosynthetic genes peptide synthase (*peps*), polyketide synthase (*pks*), non-ribosomal peptides (*nrps*) were targeted using software Geneious prime (https://www.geneious.com). Different copies of same genes present in closely related genomes of the same species has been extracted from the whole genome sequences by comparing the sequence data with database NCBI using software Geneious prime. All the gene copies were aligned together to check the nucleotide similarity. After that, the most polymorphic nucleotide region was targeted to design the specific marker on the gene sequence.

Table 4: *Streptomyces* strains used in this experiment

Strains	Host	Neighbour species	Effects in plants
EN16	Wheat	*Streptomyces griseus*	Pathogen control, growth promotion
EN23	Wheat	*Streptomyces griseus*	Pathogen control, growth promotion
EN27	Wheat	*Streptomyces roseosporus*	Pathogen control, growth promotion
CSE94	Canola	*Streptomyces ciscaucasicus*	Growth promotion
CSE46	Canola	*Streptomyces ciscaucasicus*	Growth promotion
CP200B	Chickpea	*Streptomyces cacaoi subsp. asoensis*	Nitrogen fixation, nodulation promotion, growth promotion
CP21A2	Chickpea	*Streptomyces lienomycini*	Nitrogen fixation, nodulation promotion, growth promotion
BD141	Wheat	*Streptomyces cyaneofuscatus*	Pathogen control
HCA1273	Wheat	*Streptomyces prasinosporus*	Pathogen control

2.3 Primer/marker designing

Primers designing was performed on the highly polymorphic sequences. Some features were considered for such task: forward and reverse primer sizes were set between 16-24bp and GC content between 50-65%. All designed primers were tested against the hairpin loop and other secondary structures using online the application OligoCALC (http://biotools.nubic.northwestern.edu/OligoCalc.html). Maximum score for dimer formation was set to 5 using the software Auto Dimer 1.0 (https://strbase.nist.gov). The size of the fragment to be amplified was in each strain ranged between 80 bp to 220 bp. After designing the first primer, the location of the second primers was targeted in the next 220 bp on the same gene sequence. Compatibility of selected reverse and forward primers was tested using software Auto Dimer 1.0. Primers pairs with the melting temperature close to each other were selected to validate their performance in PCR and qPCR reactions After that, the primer sequence was marked and extracted from the genes. Related information about GC content, melting temperature and sequence was retained for further use in the experiment. After designing the primers on the gene sequences, the compatibility was tested on the whole genome sequences to ensure that primer set was binding only at single target and amplify single product during PCR experiments. A set of twenty-two primers were selected (Table 5).

Table 5: Total number of primers with nucleotide sequence, fragment size, melting temperature and GC content

Sr.No.	Primer Name	Sequence	Fragment size (bp)	T_m	GC%
1	pks1 bd141 primer1(F)	CCTGGAGTACAAGATCCTGCAGTT	158	57.4	50
	pks1 bd141 primer1(R)	AGGAGCTGCACGCGGTGTCGAT		60.4	64
2	pks1 bd141 Primer2(F)	AGTACACCGTCGCGGAGACGAA	184	58.6	59
	pks1 bd141 Primer2(R)	TTGACGCCGCTGCCGAGGATCA		60.4	64
3	pks1a EN16 Primer 1(F)	GAGGTGTGCCGAGGCGGTCG	122	62	61
	pks1a EN16 Primer1(R)	GCGCCCAGCACTCGCCACC		62	66
4	pks1 EN16 Primer 2(F)	GTTCCATCACCTCGTCACGGGA	146	58	65
	pks1 EN16 Primer 2(R)	CATGCGCGAGCGGTTCGACG		60	67
5	pks1 hca Primer 1(F)	GGCGGACAGGATCGCGTTCA	207	57.9	65
	pks1 hca Primer 1(R)	CTGGACGTACACGGCATGGC		57.9	65
6	pks1 c46 Primer 1(F)	CGCTGATGCCGAACGAGGAGA	147	58.3	62
	pks1 c46 Primer 1(R)	AACAACTGCCGCGCACTCTGCA		58.6	59
7	pepsB1 c46 Primer 2(F)	GACGTACGCCGAGCTCAACTC	175	58.3	62
	pepsB1 c46 Primer 2(R)	GAAGTGCGGTTCGACGGGCAGA		60.4	64
8	pepsb5a c18 Primer3(F)	AGCGTCTCGTCGGTCACGACCA	244	60.4	64
	pepsb5a c18 Primer 3(R)	TCAGCTACGGCGAACTCGACGA		58.6	59
9	pepsB2 hca Primer2(F)	ACCGCCGCAGCACCTTTGGA	90	57.9	65
	pepsB2 hca Primer2(R)	CACCACGATCTGCGAGGGCA		57.9	65
10	nprs s94 Primer3(F)	GGAGGCGTTGCCGTATTACTGCGT	96	60.8	58
	nprs s94 Primer3(R)	AGGGCCTGCTTGTCGATCTTGC		60.8	58
11	nprs s94 Primer2(F)	ACTCCGTCTCCTCGGTCCTGGAAT	92	60.8	58
	nprs s94 Primer2(R)	GCTGACGAACGACACCAGGGT		58.2	62
12	nprs s94 Primer1(F)	ACCGTCCTGTCCGGCATCGA	125	57.9	65
	nprs s94 Primer1(R)	AGCAGTTGTGGAAGGCGGCCTG		60.4	64
13	nprs4 bd141 Primer3(F)	GTACGGGACATCGGAGCACGCT	182	60.2	67
	nprs4 bd141 Primer3(R)	AACCGGTCGATCTGGCCGAAGGC		62.4	65
14	nprs2 hca Primer3(F)	GTCTGACCTGGATGCAGGAGC	166	58.3	62
	nprs2 hca Primer3(R)	AGCTGCTCGGCGTCCTTGTG		57.9	65
15	nprs2 hca Primer4(F)	ACTTCGTGCCGTCGATGCTCG	215	58.3	62
	nprs2 hca Primer4(R)	TACAGGTTGTGCAGGCCGACAC		58.6	59
16	pks1a_c20 Primer1(F)	ACCTTCTTCTGGGCCTCGCC	138	57.9	65
	pks1a_c20 Primer1(R)	CATGCTCGTACTCGAACGGCTC		58.6	59

17	pks1b_c21 Primer2(F)	TCCAGGCTCAGCGCGACCTT	87	57.9	65
	pks1b_c21 Primer2(R)	CGGCATCGGCAGTCTGAAGG		57.9	65
18	pks1b_c21 Primer3(F)	CCAGACCGAAGGAACTGATCCC	103	58.6	59
	pks1b_c21 Primer3(R)	CGCTTCGACTTCGCCCACTC		57.9	65
19	pks1c_c21 Primer4(F)	TACAGGCGGGTGAAGGCGTCCTT	93	60.5	61
	pks1c_c21 Primer4(R)	CACCGTCACCTACATCGAGACC		58.6	59
20	pks1c_c21 Primer5(F2)	AGCCGAGCGTGATGTGCGGATC	143	60.4	64
	Pks1c_c21 primer5 (R)	CACCGTCACCTACATCGAGACC		58.6	64
21	pepsB5a_c20 Primer2(F)	TCCACTTCATCCCCGCCATGCT	139	58.6	59
	pepsB5a_c20 Primer2(R)	ACGTCGAGCAGGTCGCGGAAGT		60.4	64
22	nprs4_c21 Prime5(F)	GCTGAGGTGTCTTTCACGGTGAC	99	58.8	57
	nprs4_c21 Primer5(R)	CGGTTCCTCGCTCAGCTCGAA		58.3	62

2.4 Bacterial culture and DNA extraction

The following actinobacterial strains were grown on ISP2 media: EN16, EN23, EN27, CSE94, CSE 46, CP200B, CP21A2, BD141 and HCA1273. Pure cultures previously extracted from healthy roots were stored in glycerol (-80^{o}C). After four weeks, pure actinobacterial strains were used for DNA extraction using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method. For each strain, approximately 0.5 g bacterial colonies transferred into 800 µl modified CTAB buffer along with 0.5g of 0.1 Micron Zirconium beads (Bio spec products). Subsequently, 800 µl of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma Aldrich) was added to the suspension. Tubes were shaken in bead beater instrument (Bio spec products) for 5min followed by incubation for 1h at 65^{o}C using water bath instruments. After that, centrifugation was done at 16000rpm for 5min at 4^{o}C. The upper aqueous layer was retained to the new tube and the same volume of chloroform-isoamyl (24:1) (Sigma Aldrich) was added to the retained upper layer. In the next step, tubes were centrifuged at 16000rpm for 5min at room temperature followed by transferring the top layer into the new tube. Two volumes of Polyethylene glycol/ sodium chloride precipitate solution were added and incubated for 2h at 4^{o}C. The solution was centrifuged at 16000rpm for 15min at 4^{o}C for pellet formation. The supernatant was gently poured off without disturbing the pellet. Furthermore, the washing of

pellet was conducted twice using chilled 70% ethanol with centrifugation for 10min at 16000rpm. The supernatant was removed without disturbing pellet. In last, DNA pellet was resuspended into the 50ml MiliQ water. DNA was quantified using Nanodrop (Thermo Fisher 2000/2000) and further dilutions were made for PCR application.

2.4.1 Optimization of primers using conventional PCR

All designed primer pairs chosen from in silico methods were optimised using conventional PCR. Twenty-two primer pairs were tested on their *Streptomyces* genomes to get desired amplification of selected nucleotide sequences. After that, the specificity of each primer was tested against other *Streptomyces* genomes. Optimization of primers was achieved using different temperatures to get specific fragment amplification only in a single strain.

PCR was carried out with 10 µl reaction mixture. The final 10µl reaction mixture was made using 5µl 2x Taq Master mix, 0.2µl 25mM $Mgcl_2$ (Biolabs), 3µl nuclease-free water, 0.4µl forward primer, 0.4µl reverse primer and 1µl bacterial DNA (20 ng/µl). The temperature and cycle profile for the PCR reaction was set as Table 6.

Table 6: Thermocycler profile for PCR (polymerase chain reaction)

Steps	Temperature	Time (minutes)	Cycles
Pre denaturation	94 ^{o}C	3	1
Denaturation	94 ^{o}C	1	30
Annealing	55 ^{o} to 70 ^{o}C*	1	30
Extension	72 ^{o}C	2	30
Final extension	72 ^{o}C	10	1

* Annealing temperature changes according to the melting temperature of the primer and to optimise the PCR specificity temperature changes to increase the specificity of the primer.

The risk for DNA damage and amplification of unwanted products increases with a higher number of cycles (Cannon et al. 2019; Eckert et al. 1991). Primary reagents used in nucleic acid purification (salts, guanidine, proteases, organic solvents and SDS) are major inhibitors for DNA polymerases like phenols (Katcher et al. 1994).

2.4.2 DNA extraction from shoot, root seed and soil

DNA from various seeds, root and soil samples were extracted to determine the presence and quantification of the selected bacterial strain in the sample. Seeds of selected samples were previously inoculated with *Streptomyces* strains. Plant and soil samples without seed inoculation were also chosen to use them as control. Soil and root samples were selected from diseased (*Rhizoctonia* affected) and non-diseased plants to compare the difference. Plants with disease and without disease were selected from week 4, 8, 12 and 20 for strain EN16 and HCA1273. Root and soil samples were collected from the field and glasshouse trials at different period of growth to test the specificity of developed markers and tracking of *Streptomyces* strain, i.e. EN16 and HCA1273. All the soil and plant were extracted using the same modified CTAB method. Chickpeas coated with CP200B were planted in the glasshouse for eight weeks and the DNA from shoots, roots and rhizosphere soil were extracted after week 4 and week 8 using the same extraction method. Along with that, DNA from the chickpea plants grown without application of CP200B was extracted at week 4 and 8.

2.4.3 Quantification of DNA using real-time qPCR

Real-time quantitative PCR was performed with 96 well-plates (BioRad) in a CFX96 real-time qPCR system (BioRad) to conduct the experiment. The reaction was performed with 10µl DNA, 2µl of diluted forward and reverse primer mix (5µM), 5µl Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2µl water. Non-template controls were used with DNA being replaced by water in these samples. The qPCR reactions were performed as follows:

95°C for 2 min, 40 cycles of; 95 °C for 15 seconds, 60°C for 1 minute. A melt curve was performed at the end with 58 °C for 5 seconds and 95°C for 15 seconds. PowerUp™ SYBR® green ensures the sensitivity, reproducibility and detection of low copies of DNA present in different template concentration (https://www.thermofisher.com). The number of cycles were selected to the maximum 40 to reduce the amplification of unwanted targets. Annealing temperature performs a critical role in the amplification of a specific target in qPCR. The annealing temperature for each selected primer for qPCR assay was optimised using conventional PCR to get specific fragment amplification. The annealing time was set as 30 sec to reduce the unspecific annealing and amplification. Intial 10-15 cycles of qPCR reaction produce backround noices, there is very little change is detectable in these cycles. The fraction number of cycles (C) are required to produce a fluorescent signal exceeding the threshhold (t) (Platts et al. 2008). This particular cycle where flourescence signal crosses the threshold is known as threshold cycle (Ct value).

2.5 Data analysis

All tests were performed at least 2 replicates and the values were entered and collated in an MS Excel spreadsheet. The data was analysed and shown with mean and standard error (SE). T-test was performed to detect statistical significance, and $P < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 AntiSMASH analyses

AntiSMASH was used to monitor the diversity of gene clusters for metabolite production between closely related strains using the whole-genome sequences for 9 strains. Different gene clusters or part of the gene clusters present in submitted *Streptomyces* genome produces diverse secondary metabolites. Then the number of single type gene clusters present in each strain were calculated (Figure 3).

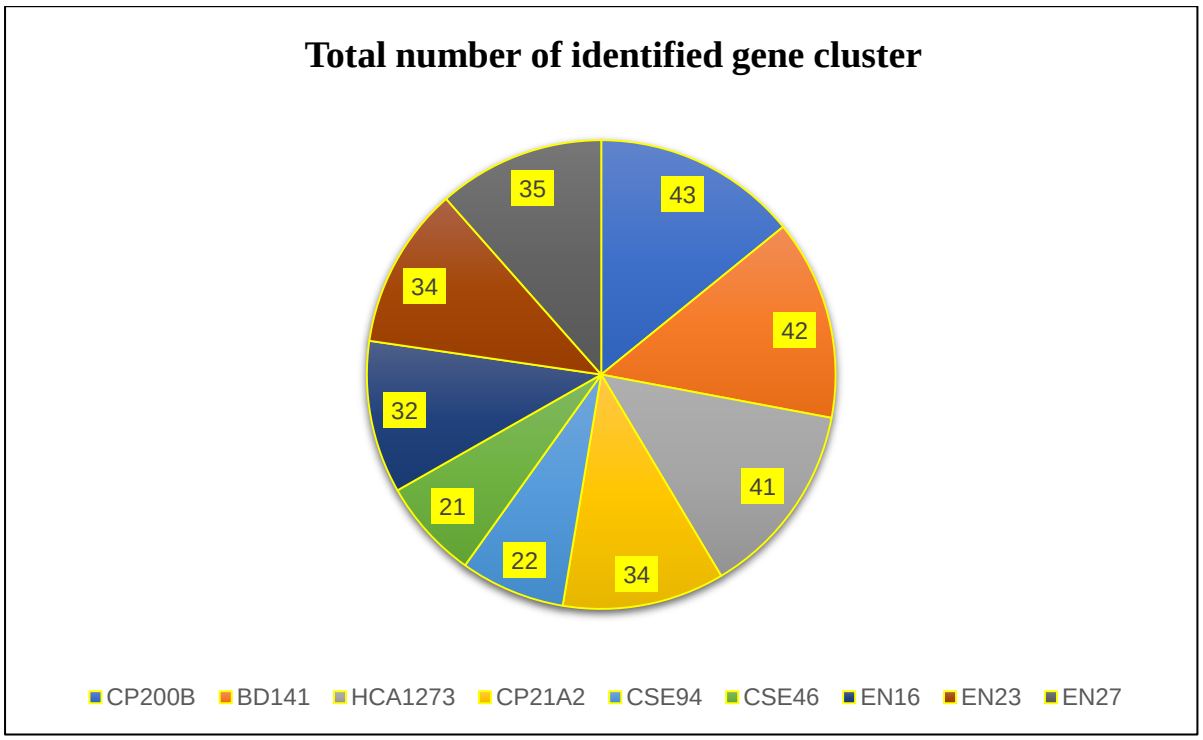


Figure 3: Total number of 304 gene clusters were identified in nine *Streptomyces* strains using AntiSMASH. Highest number of gene clusters were identified in the genome of strain CP200B and BD141 i.e. 43 and 42, respectively. Strain CSE46, CSE 94 reflected 21 and 22 gene clusters' respectively. Genomic sequence of strain EN16, EN23, EN27, HCA1273 revealed 32, 34, 35 and 34 gene clusters. Respectively; - 34 gene clusters were recognised in the genome sequence of strain CP21A2.

The type of gene clusters present was examined to understand the diversity of each gene cluster present in the different *Streptomyces* genomes. Most of the selected strains reveal the diversity

of some common gene cluster present in all the strains i.e. NRPS, Terpenes (Figure 4). Along with this, some of the gene clusters were present only on specific strains only like thiopeptide, hglE-KS.

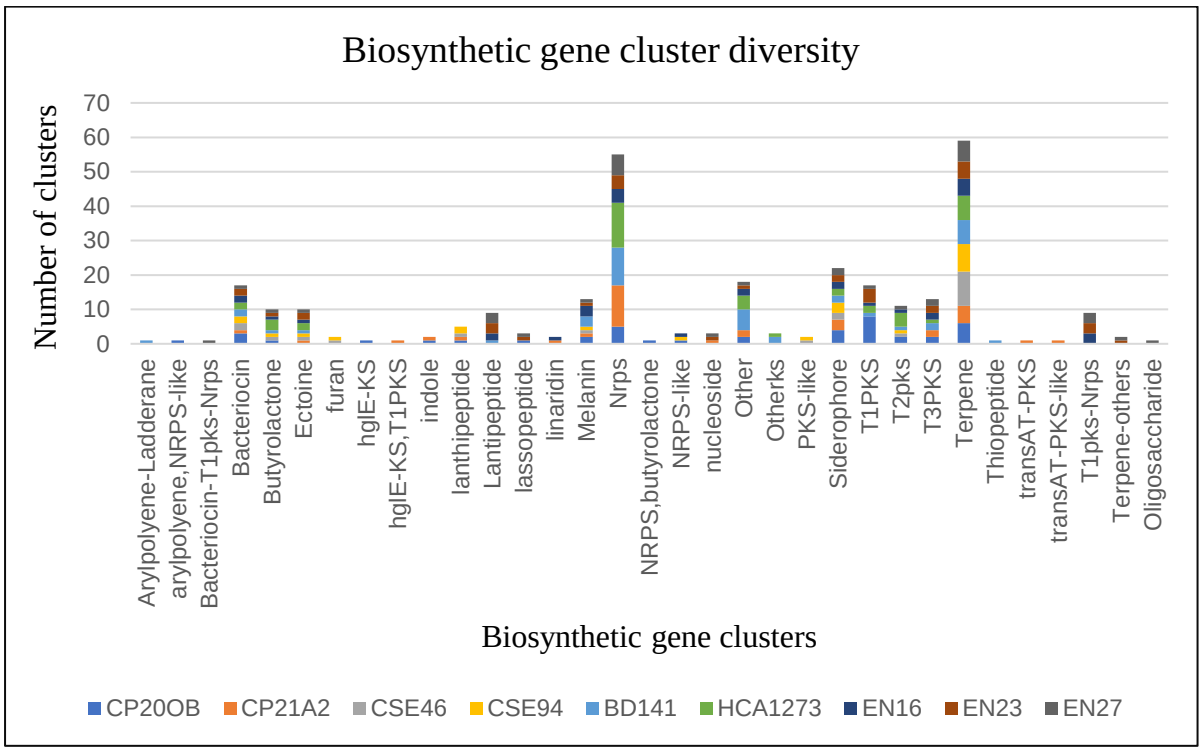


Figure 4: Numerical comparison of biosynthetic gene clusters present in genomes of nine selected strains. EN16, EN23, EN27, C20, C21, C46, C94, BD141, HCA1273 using antiSMASH analysis tool. Gene cluster type bacteriocin, terpenes and siderophores were identified in all the selected nine strains. Total of 109 gene clusters were recognized as non-ribosomal peptides synthases (NRPS) and terpenes shared by all selected *Streptomyces* strains. Bacteriocin, ectoine, melanin, siderophore and T3PKS were noticed in at least seven selected strains. Aryl polyene-Ladderane and hglE-KS gene clusters were only seen in strain BD141 and CP200B respectively.

Some of the metabolites like hopene were shared by all the strains used in this experiment (Figure 5). Similarly, some strains contain unique metabolites in their genome sequences i.e. flaviolin in HCA1273.

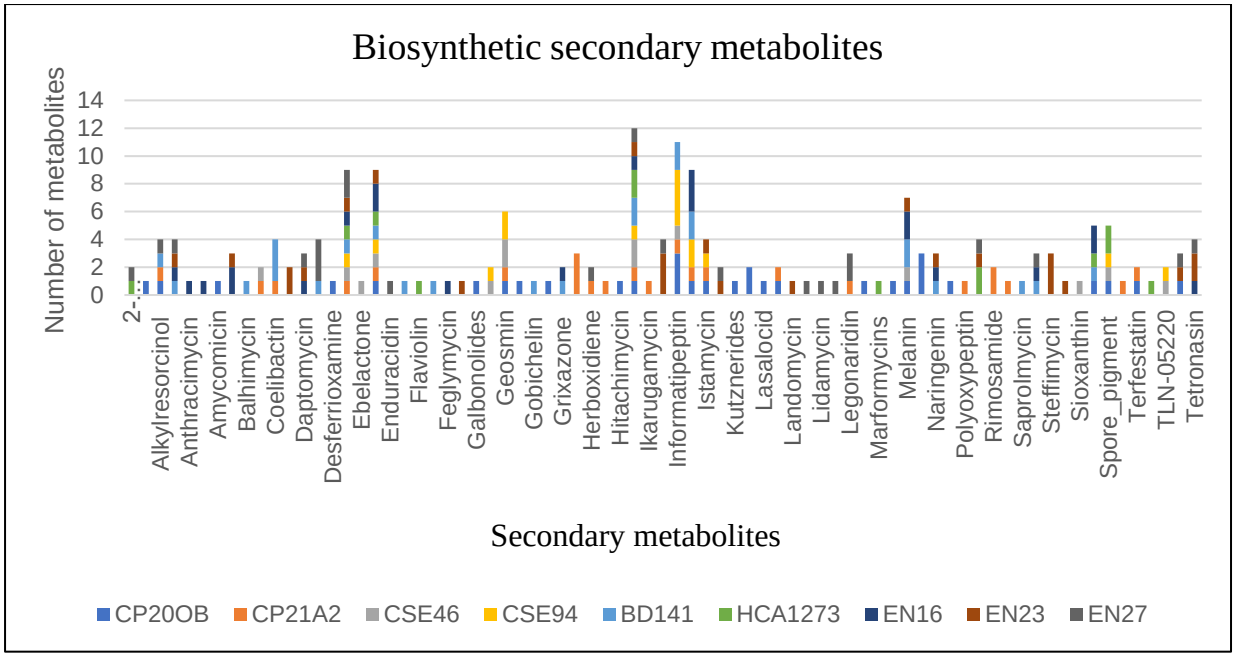


Figure 5: Distribution of secondary metabolites among 9 *Streptomyces* strains. Hopene was reported in every single strain tested in antiSMASH for this experiment with the highest number (12) of metabolites. Metabolite isorenieratene, ectoine, desferrioxamine_B, informatipeptin were present in at least six selected strains. Melanin and albaflavenone reveal their presence in at least five strains. Existence of secondary metabolite skyllamycin, spore pigment, AmfS was recognised in at least four strains. Taromycin, flavlolin, anthracimycin, hitachimycin were only identified in single strains.

3.2 Alignment of gene sequences to find unique polymorphic region

Along with housekeeping genes i.e. *atpD, gyrB, recA, rpoB* and *trpB,* other sequences of 15 genes (*MethH, tafA, prcA, dnaK, hrdB, sigB, whiG, shbB, rbpA, trxA, trxB, hrdC, lcp)* were selected from the *Streptomyces* to find unique polymorphic region in single strain from same species. The various species of *Streptomyces* were targeted to find unique polymorphic regions. Most of the gene sequences present in the database for *Streptomyces* present in different strain gave similar nucleotide sequences after alignment (Figure 6). Some polymorphic regions were found in some alignments, but it was not enough to design sets of primer according to the

requirement of experiment i.e. fragment sizes, dimer formation, size of primer, GC content of *Streptomyces* spp. in figure below.

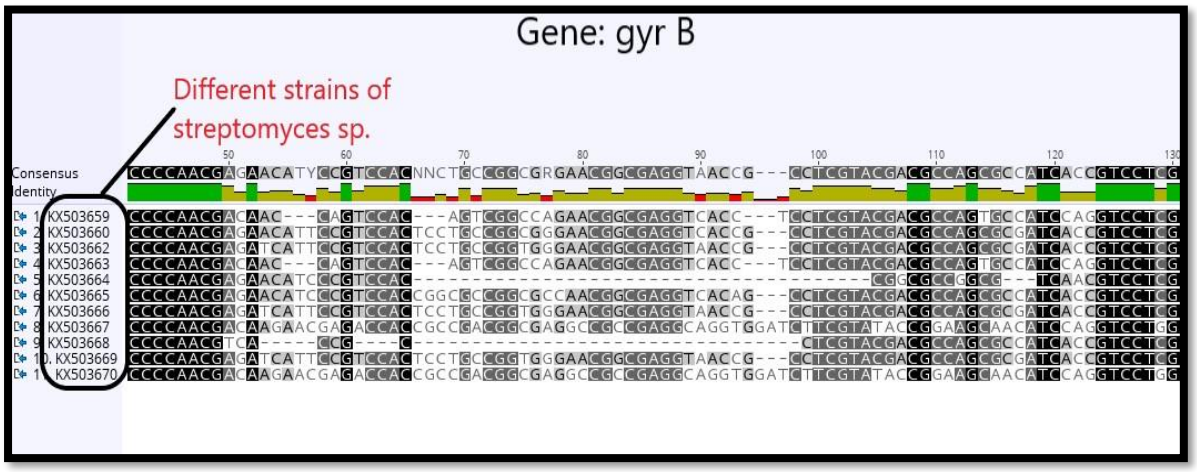


Figure 6: Alignment of gene sequences from gyr B gene present in different *Streptomyces* strains using software Geneious prime**.** The black region in the figure represents the similarity between nucleotide sequences. Grey and white nucleotide region represent the polymorphic region.

Figure 6 represents the average polymorphic regions found in all other gene sequences as well. Some nucleotide sequences like in the last gene sequence of alignment showed polymorphic nucleotides, but they were present in at least one other strain. This approach was followed to find a unique region in other *Streptomyces* strain and look for the same region in other strain.

3.3 Alignment of transporter biosynthetic genes and detection of the polymorphic region

After negative results to find a polymorphic region in housekeeping and other conserved genes, a new approach was conducted to target multiple copies of transporter genes in the selected genomes. AntiSMASH results depicted that some pathways are common in all of the strains, i.e. nrps and pks. This data leads us to target some conserved genes present in these strains. All the copies of transporter genes, i.e. *nrps, pepsB, pks* were extracted from targeted *Streptomyces*

strain and closely related strains. Alignment of all the gene copies gave some suitable polymorphic regions to design strain-specific primers in single strain (Figure 7). Polymorphic regions present only in the genome of a single strain were targeted to design both forward and reverse primers.

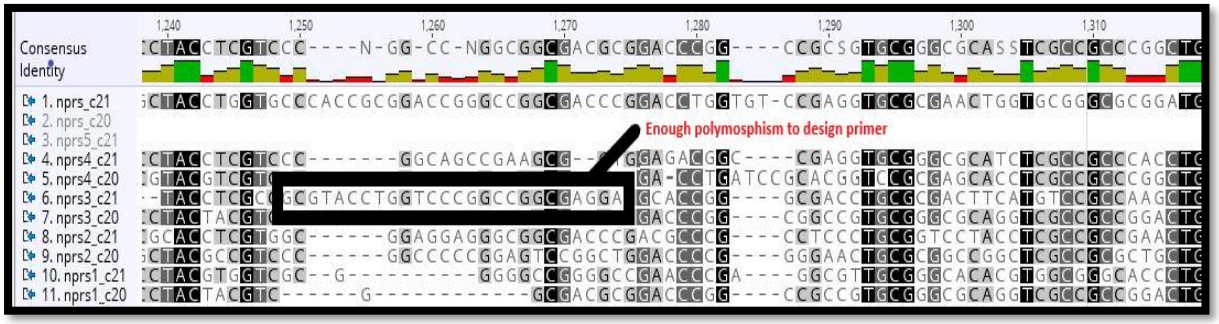


Figure 7: Polymorphic region detected after alignment of multiple copies of the same gene present in *Streptomyces* strain CP200B and CP21A2. Polymorphic nucleotide sequence shown in figure filled the requirement, i.e. GC content, size, melting temperature, dimer check to design a primer.

Primers with mandatory features were designed on the gene sequences and then tested on the whole genome sequences of *Streptomyces* strains to check compatibility (Figure 8). Primers binding at single target to amplify desired fragment has been selected.

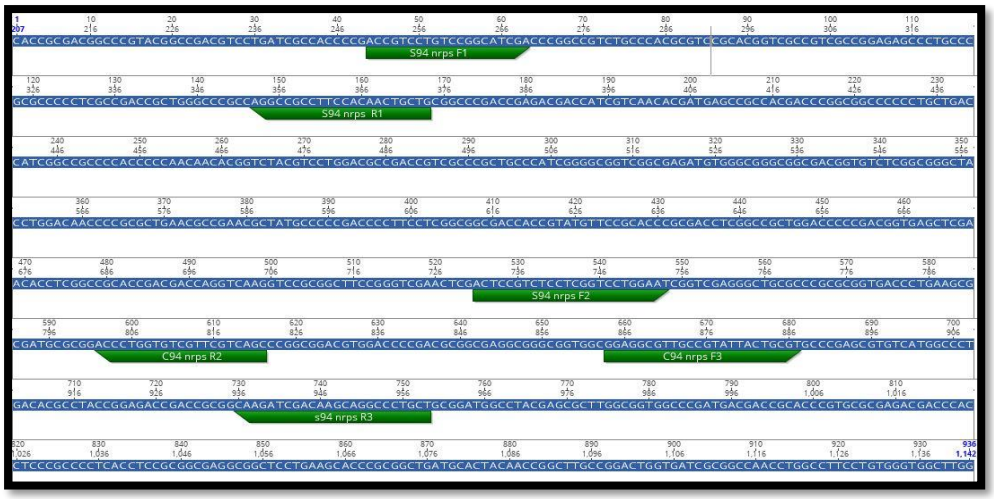


Figure 8: A Set of Forward and reverse primers designed on the highly polymorphic nucleotide region to amplify short fragments on the genome of *Streptomyces* strain CSE94. All the primers were sized in between 16 to 24 bp. The size of the fragment to be amplified was not more than 220 bp.

3.4 PCR for specificity on different strains

Firstly, a single set of primers was tested on the *Streptomyces* strain for which it had been designed. After getting positive amplification of the same bp fragment on which specific primer had been designed, it was further tested on the closely related and available *Streptomyces* strains to check the specificity. There is a possibility of binding a designed primer to some other nucleotide sequences in the genome and amplification of the unspecific fragment. A single primer pair 21(Table 5) for CSE46 amplified the same fragment in the closely related strain CSE94 (Figure 9). Positive amplification on the *Streptomyces* strain's genome and no amplification on the closely related strains have been achieved to screen the strain-specific primers (Figure 9).

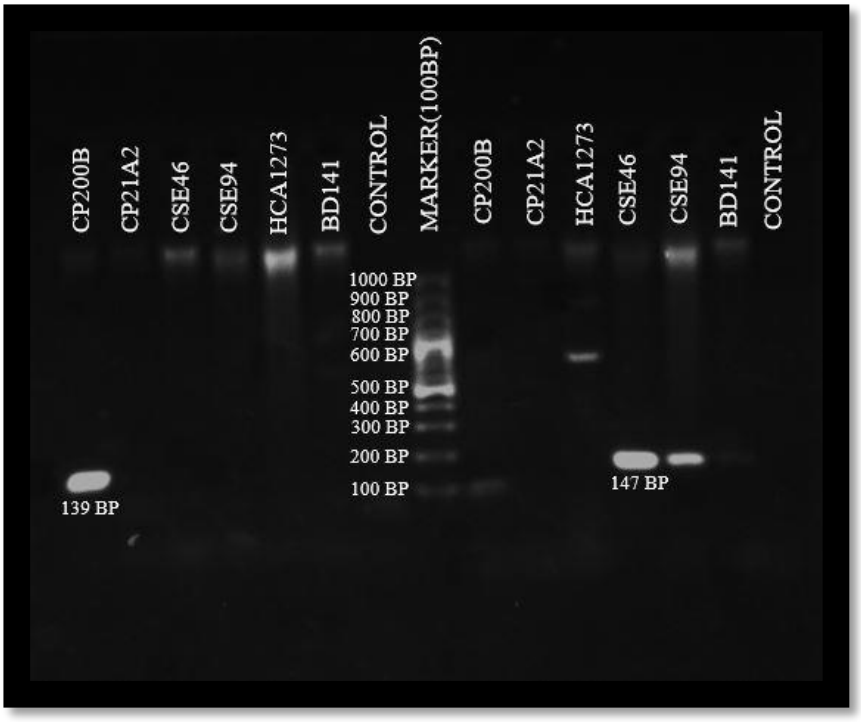


Figure 9: PCR amplification of 139 bp fragment on genomic DNA of *Streptomyces* strain CP200B using strain-specific primer set 21 (Table 5) and negative amplification results on closely related *Streptomyces* strains with the same set of primer (CP21A2, CSE46, CSE94, BD141, HCA1273). PCR amplification of 147 bp fragment on genomic DNA of *Streptomyces* strain CSE46 using primer set 6 (Table 5) and positive amplification results on genomic DNA of strain CSE94 along with unspecific amplification in strain HCA1273. Control sample represents the negative control without any DNA.

For strain HCA1273, primer set 9 and 14 (Table 5) showed positive amplification with fragment size 90 and 166, respectively on the pure culture DNA of strain HCA1273. Both sets of primers showed no amplification on closely related strain BD141 and other related *Streptomyces* strains, i.e. CP200B, CP21A2, CSE94, CSE 46. In strain BD141, primer set 2 and 13 amplified positive fragments with size 184 and 182 bp respectively on DNA from pure culture BD141. Similarly, both primers have not amplified any fragment on related *Streptomyces* genomes, i.e. HCA1273, CP200B, CP21A2, CSE94 and CSE46. Total of six

strain-specific markers was developed at the end of a conventional PCR experiment. Primer

Table 7: Optimization of Primers on DNA of pure culture using conventional PCR

	T_m (°C)	Fragment size on gel (bp)						
Streptomyces strain (Primer set)			CP200B	CP21A2B	CSE46	CSE94	BD141	HCA1273
CP200B (1)	58	138	+	*	*	*	-	-
CP200B (2)	59.5	139	+	-	-	-	-	-
CP21A2B (1)	58	87	+	+	-	-	*	*
CP21A2B (2)	58	103	+	+	-	-	*	*
CP21A2B (3)	58	93	+	+	-	-	*	*
CP21A2B (4)	58	143	+	+	-	-	-	-
CP21A2B (5)	58	99	+	+	-	-	-	-
CSE46 (1)	59.5	147	-	-	+	+	-	-
CSE46 (2)	60	175	-	-	+	+	*	*
CSE94 (1)	58	125	-	-	*	+	*	*
CSE94 (2)	60	92	-	-	+	+	-	-
CSE94 (3)	60	96	-	-	+	+	*	-
BD141 (1)	58	158	+	+	+	+	+	-
BD141 (2)	58	184	-	-	-	-	+	-
BD141 (3)	72	182	-	-	-	-	+	-
EN16 (1)	60	122	+ (EN16)	-	-	-	-	-
EN16 (2)	59	146	+ (EN16)	*	-	-	-	-
HCA1273 (1)	58	207	-	-	*	*	+	+
HCA1273 (2)	58	90	-	-	-	-	-	+
HCA1273 (3)	60	166	-	-	-	-	-	+
HCA1273 (4)	60	215	*	+	*	*	*	+

(+) : Positive amplification of expected fragment, (-) : No amplification, (*) : Non specific Amplification

set number 21 (CP200B), 9 and 14 (HCA1273), 2 and 13 (BD141), 3 (EN16) were selected as strain-specific primers after checking the negative amplification on selected closely related strains (Table 7). Starting from 5°C below than T_m and then increased to determine the best annealing conditions appropriate optimisation of primer. Use of slightly higher annealing temperature can minimise the amount of unspecific PCR product (Sambo et al. 2018)..

3.5 Quantification of CP200B in the root, shoot and soil in chickpea

Dilution series using pure culture DNA with ratio 1:10 was made and quantified. Using the same dilution series qPCR was conducted and Ct values were retained (Figure 10). A standard curve was plotted using the log concentration of dilution series and Ct value from qPCR (Figure 11). This standard curve was used to get the DNA concentration present in the different samples taken from the different sources.

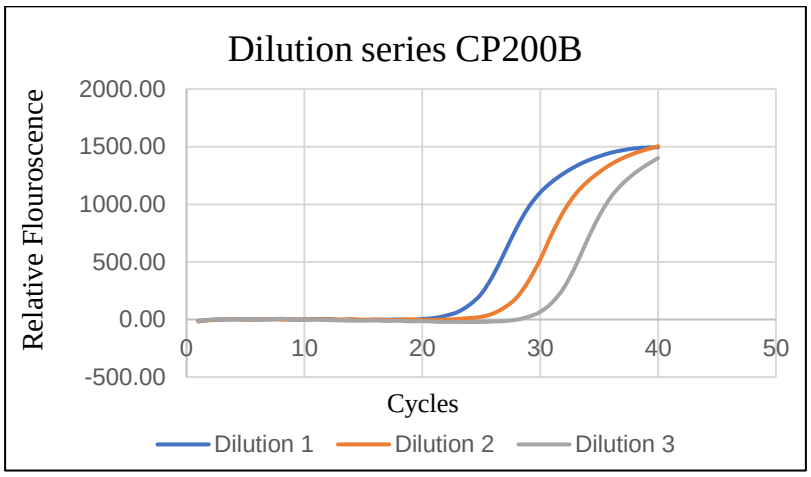


Figure 10: Serial dilutions 1:10 using primer set 21 (Table 5) on pure culture genomic DNA of strain CP200B

Table 8: Log DNA concentration and Ct values of dilution series from CP200B

DNA Dilution	Log10 DNA amount (ng)	Ct value
Dilution 1	0.99	22.01
Dilution 2	0.67	27.95
Dilution 3	0.398	35.33

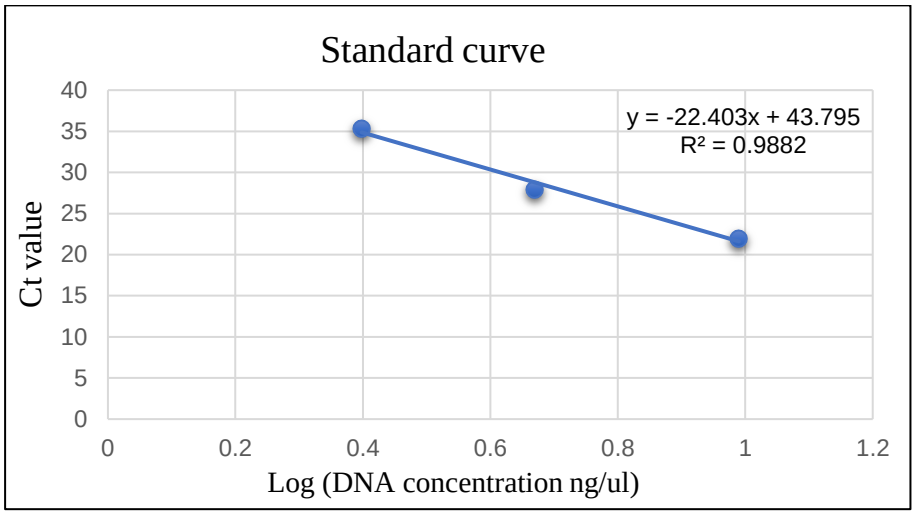


Figure 11: Correlation between Log (DNA conc. Ng/ul) and Ct value for dilution series. Efficiency (R2) for Primer binding each cycle is 0.9882.

DNA from the soil samples were tested against standard dilutions in replicates using selected primer set 21 (Table 5) to quantify the strain CP200B in soil and rhizosphere of chickpea plant under glasshouse trails. Six samples from soil were tested in quantitative PCR to check the presence of strain CP200B in soil and rhizosphere at different growth periods. At week 4, the presence of strain CP200B in the rhizosphere and bottom pot soil was detected using a strain-specific primer (Figure 12 and Table 9). Unspecific detection was also observed in the rhizosphere of a plant untreated from CP200B at week 4. Untreated plant is considered as the sample taken from the plant not grown under CP200B application. Out of 6 samples, four samples revealed amplification with the primers designed for CP200B, and two samples showed no detection of strain CP200B. Week 8 rhizosphere soil resulted in most DNA concentration (Table 9). Rhizosphere soil from the plant pot (untreated with CP200B) at week four also revealed non-specific amplification with the primers designed for CP200B. The presence of CP200B was detected in the rhizosphere soil collected after 4weeks of plant growth (Table 9) respectively. Soil from the bottom of the pot at week 8 was unable to display the

presence of strain CP200B. No unspecific amplification with the primers designed for CP200B was observed in the plant roots (Untreated from CP200B) at week 8.

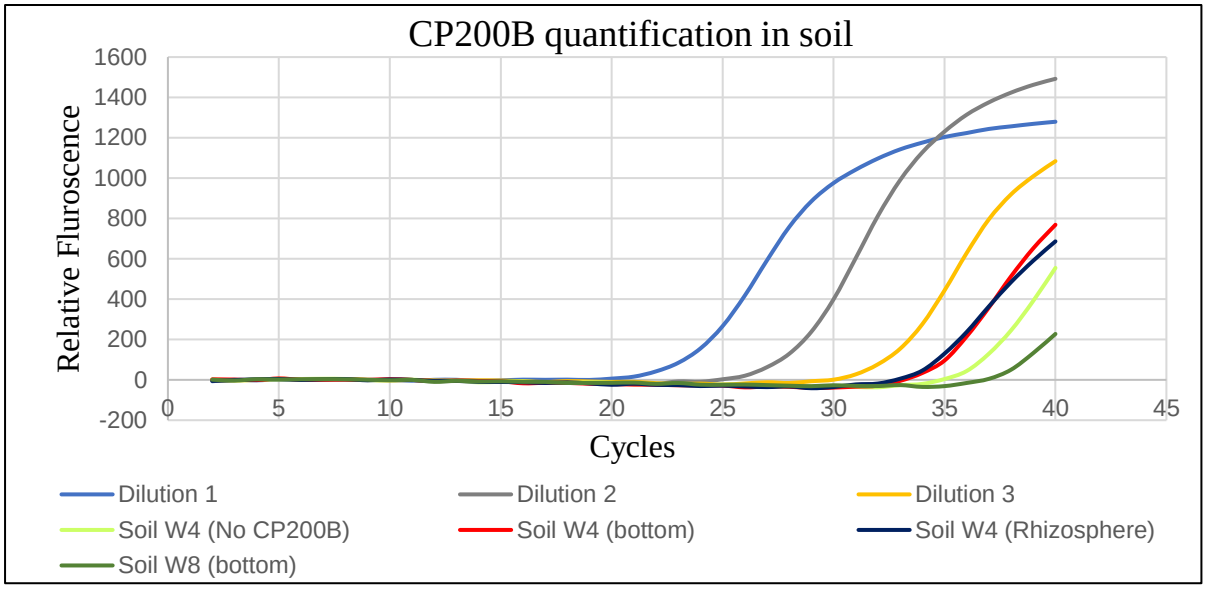


Figure 12. Quantification of *Streptomyces* strain CP200B in soil and rhizosphere of chickpea plant (Glasshouse) at week 4 and week 8 using designed primer set 21 (Table 5). Soil from week 4, both rhizosphere and bottom resulted in more DNA presence. No DNA was detected in the rhizosphere at week 8.

Note: No CP200B- Sample from a plant grown without CP200B application, CP200B - Sample from a plant grown under CP200B application, Dilution 1,2,3 – Dilution series (1:10) DNA from pure culture.

Table 9: DNA concentration and Ct value of CP200B in soil samples

DNA Sample (Soil and rhizosphere)	Ct value	DNA concentration (ng/g soil)
Rhizosphere soil week 4 (No CP200B)	35.89	0.74 ±0.06
Rhizosphere soil week 8 (No CP200B)	N/A	N/A
Rhizosphere soil week 4 (CP200B)	34.09	0.87±0.01*
Bottom soil week 4 (CP200B)	33.84	0.89±0.05**
Rhizosphere soil week 8 (CP200B)	32.87	0.96±0.005**
Normal soil week 8 (CP200B)	N/A	N/A
Negative control (No DNA)	N/A	N/A

Note: No CP200B- Sample from a plant grown without CP200B application, CP200B - Sample from a plant grown under CP200B application, N/A – No amplification, ± - Standard error for replicates, *-the significant differences between the treatments (*P<0.05, **P<0.01, T-test).

Quantitative PCR disclosed the quantity of strain CP200B present in the DNA of roots and shoots at different period of growth, i.e. 4 and 8 weeks (Figure 13). Four DNA samples were taken from the root and shoot of the plants grown without application of strain CP200B. Two samples of each, i.e. root and shoot DNA from the plant grown under the application of *Streptomyces* strain CP200B were tested with designed specific primer. DNA samples from shoot and root at week 8 exposed the occurrence of CP200B in high amount (Table 10). Detection of strain CP200B was not seen in the control shoot samples (CP200B untreated) for weeks 4 and 8. The DNA sample from the control root (CP200B untreated) at week 4 also reveals some unspecific amplification (Table 10). There was no evidence for the occurrence of non-specific amplification with the primers designed for CP200B in the control shoot (Untreated CP200B) of week 4.

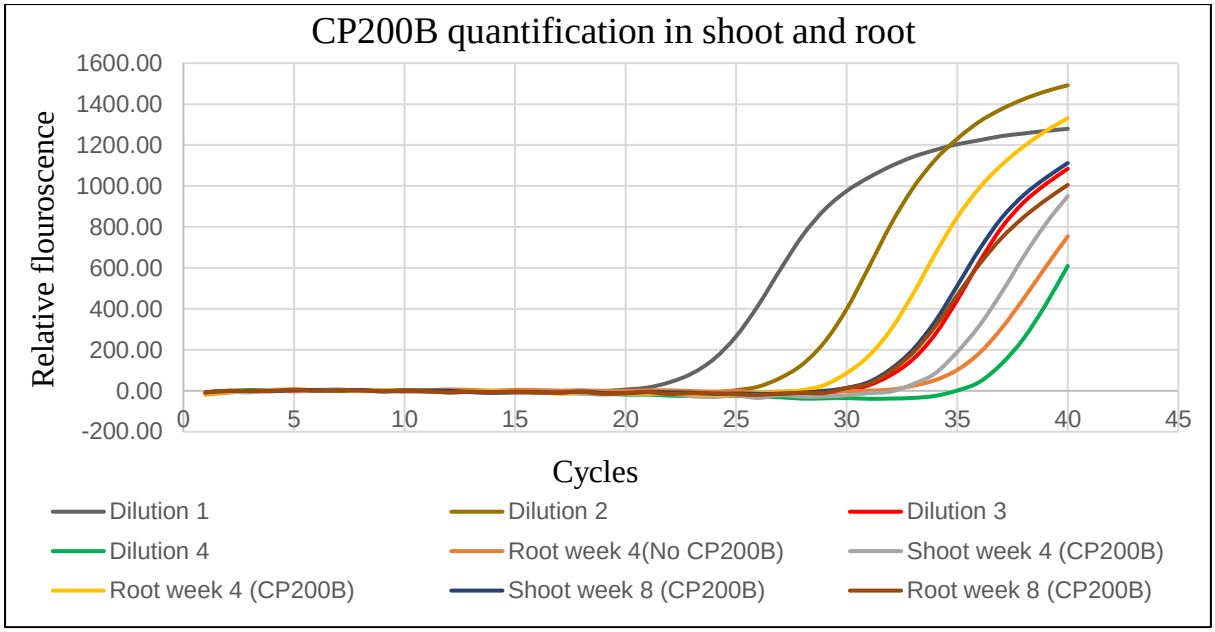


Figure 13: Quantification of Streptomyces strain CP200B in root and shoot of chickpea plant (Glasshouse) at week 4 and week 8 using designed primer set 21 (Table 5). Quantitative PCR results reveal the highest amount of *Streptomyces* strain CP200B (Table 10) is present in the roots of chickpea plant at week 4. In week 4, DNA from root also displays the occurrence of (Table 10) in plant untreated with CP200B.

Note: No CP200B- Sample from a plant grown without CP200B application, CP200B - Sample from a plant grown under CP200B application, N/A – No amplification, Dilution 1,2,3 – Dilution series (1:10) DNA from pure culture.

Table 10: DNA concentration and Ct value of CP200B in root and shoot samples

DNA sample (shoot and root)	Ct value	DNA Concentration (ng/g plant part)
Shoot week 4 (No CP200B)	N/A	N/A
Root week 4 (No CP200B)	35.3	0.76±0.07
Shoot week 4 (CP200B)	34.32	0.85±0.007*
Root week 4 (CP200B)	30.49	1.19±0.01***
Shoot week 8 (No CP200B)	N/A	N/A
Root week 8 (No CP200B)	N/A	N/A
Shoot week 8 (CP200B)	32.24	1.03±0.005*
Root week 8 (CP200B)	32.35	1.02±0.005*
Negative control (No DNA)	N/A	N/A

Note: No CP200B- Sample from a plant grown without CP200B application, CP200B - Sample from a plant grown under CP200B application, N/A – No amplification, ± - Standard error for replicates, *-the significant differences between the treatments (*P<0.05, ***P<0.001, T-test).

3.6 Quantification of strain EN 16 in roots, seed and soil of wheat

Total different 14 samples were selected for qPCR. DNA from the EN16 coated seeds showed the highest concentration with Ct value 23.68 in qPCR results (Figure 14, Table 11). Root sample (EN16 treated) from week 12 revealed the presence of 0.617587 ng/g DNA of strain EN16 (Table 11). Root Sample from week 12 (EN16 untreated) depicted non-specific amplification (Figure 14). None of the control soil (EN16 untreated) samples reflects the amplification of EN16. Soil from week 4 displays a high amount of EN16 in the rhizosphere (Table 11). Similarly, low amount of EN16 with Ct value 34.94 was detected in the rhizosphere soil of *Rhizoctonia* affected plants. At week 20, soil from the rhizosphere of non-diseased plant depicts less amount of EN16 with Ct value of 32.68 and 30.44. *Rhizoctonia* infested soils for week 20 showed a 33.62 Ct value (Table 11).

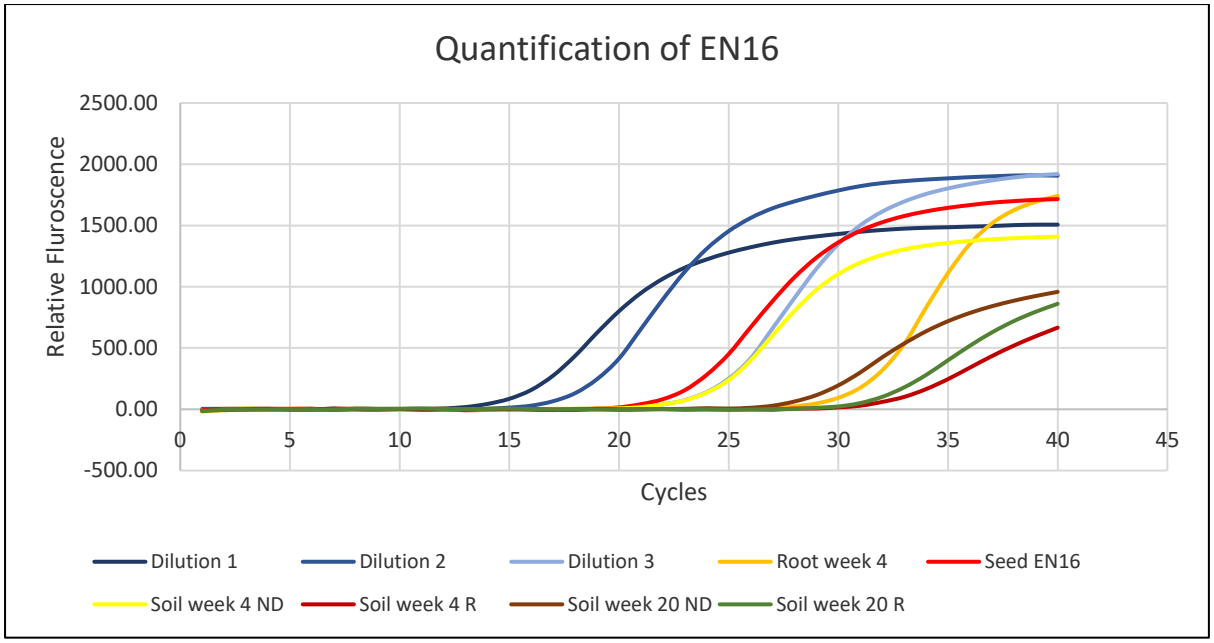


Figure 14: Quantification of Streptomyces strain EN16 in seed, root and soil samples from Glasshouse using primer set 3 (Table 5). Seed DNA (positive control) contains the highest amount of strain EN16. Soil from week 4 resulted in a high amount of EN16. Soil from the rhizoctonia affected plant at week 4 showed the lowest amplification in the qPCR test. No amplification was seen in the any of control soil sample (No EN16).

Note: R- *Rhizoctonia* present in the roots, ND- No disease present in the roots, No EN16- Sample from a plant grown without EN16 application, EN16- Sample from a plant grown under EN16 application, Dilution 1,2,3 – Dilution series (1:10) DNA from pure culture.

Table 11: DNA concentration and Ct value for strain EN16 in the root, seed and soil

DNA sample (Seed, root and soil)	Ct value	DNA Concentration (ng/g sample)
Seed EN16 treated (Positive control)	23.68	1.86±0.05
Root week 12 (EN16) [ND]	31.46	0.62±0.03
Control Soil (No EN16)	N/A	N/A
Control Soil (No EN16)	N/A	N/A
Control Soil (No EN16)	N/A	N/A
Control Soil Week 20 (No EN16) [R]	N/A	N/A
Control Soil Week 20 (No EN16) [ND]	N/A	N/A
Soil Week 4 (EN16) [ND]	25.02	1.60±0.02***
Soil Week 4 (EN16) [ND]	29.22	0.97±0.02***
Soil Week 4 (EN16) [R]	34.94	0.06±0.01**
Soil Week 20 (EN16) [ND]	32.68	0.42±0.005**
Soil Week 20 (EN16) [ND]	30.44	0.78±0.02***
Soil Week 20 (EN16) [R]	33.62	0.27±0.003**
Negative Control (No DNA)	N/A	N/A

Note: R- *Rhizoctonia* present in the roots, ND- No disease present in the roots, F- Field sample, No EN16- Sample from a plant grown without EN16 application, EN16- Sample from a plant grown under EN16 application, N/A – No amplification, ± - Standard error for replicates, *-the significant differences between the treatments (**P<0.01, ***P<0.001, T-test).

3.7 Quantification of *Streptomyces* strain HCA1273 in the seed, root and soil samples of wheat

For *Streptomyces* strain HCA1273, seed sample coated with HCA1273 was used as a positive control. Root and soil samples were selected from both HCA173 treated and untreated plant. Seed DNA revealed positive amplification in the qPCR reaction (Figure 15). Control root (Untreated) sample from week 4 showed the presence unspecific strains (Table 12). HCA1273 treated plant from week eight also depicts the presence of low amount of HCA1273 with Ct value 38.14. None of the soil samples i.e. treated or untreated with HCA1273 from weeks 4 or 20, reflected any significant amplification in the experiment.

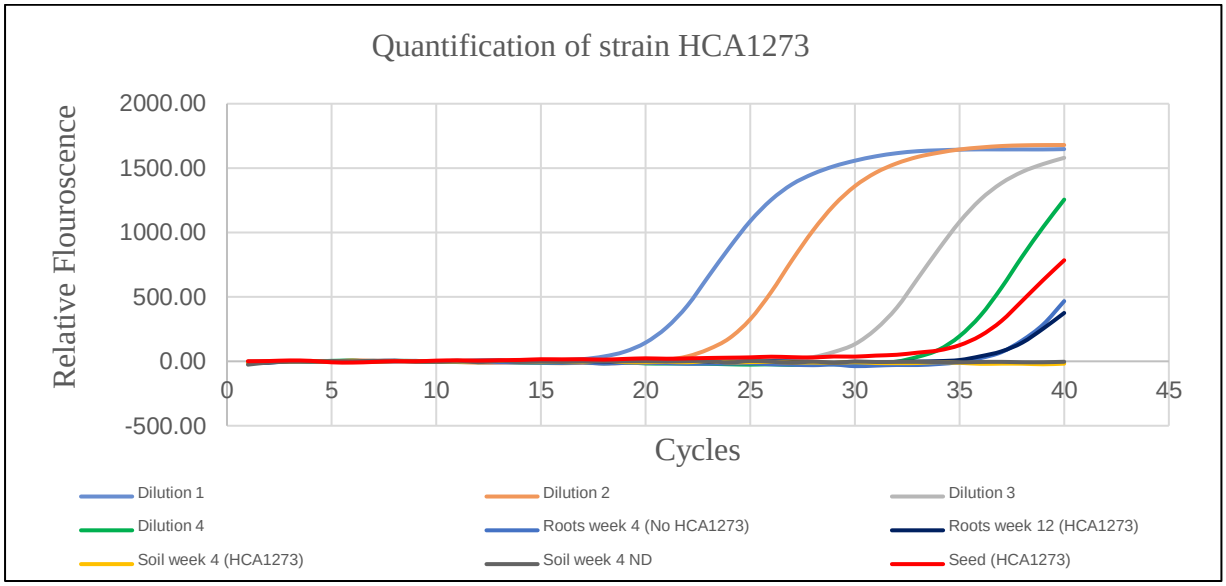


Figure 15: Quantification of HCA 1273 in seed, root and soil samples using primer set 14 (Table 4)

ND- No disease present in the roots, No HCA1273- Sample from a plant grown without HCA1273 application, HCA1273- Sample from a plant grown under HCA1273 application

Table 12: DNA concentration and Ct value for strain HCA 1273 in root, seed and soil.

Plant and root sample	Ct value	DNA concentration (ng/g sample)
Seed (HCA1273) Positive control	35.48	0.27±0.001
Root (No HCA1273)	37.97	0.02±0.005
Root week 8 (HCA1273)	38.14	0.0005±0.01
Control soil (No HCA1273)	N/A	N/A
Soil week 4 (HCA1273) [ND]	N/A	N/A
Soil week 4 (HCA1273) [R]	N/A	N/A
Soil week 20 (HCA1273) [ND]	N/A	N/A
Soil week 20 (HCA1272) [R]	N/A	N/A
Negative Control (No DNA)	N/A	N/A

Note: R- Rhizoctonia present in the roots, ND- No disease present in the roots, No HCA1273- Sample from a plant grown without HCA1273 application, HCA1273- Sample from a plant grown under HCA1273 application, N/A – No amplification, ± - Standard error for replicates, no significance between the treatments.

CHAPTER 4

DISCUSSION

4.1 In silico primer optimisation using bioinformatic tools

Selection of genes for strain tracking depends upon the general features like ease of amplification, previous analyses of closely related taxa, rate of evolution, specificity and copy number (Guo et al. 2008). Apart from this, the selected gene must have enough polymorphism in sequence to differentiate one strain from the diversity of all other bacterial strains. A low percentage of polymorphism will manifest the probability of giving negative results for discrimination of strains. Transporter biosynthetic genes *pks, peps, nrps* were targeted for alignment and primer designing because it could respond to all these requirements.

Primer design is generally aimed to target two goals, i.e. specificity and efficiency. Poor specificity may give false results in the DNA fragment amplification. Primers sized between 18 to 24 bp oligonucleotides are generally considered to be sequence-specific (Dieffenbach et al. 1993). Longer primers take more time to hybridize, extend and a longer time to remove (Thornton et al. 2011). Melting temperature (T_m) of the primer generally falls in the range of 55- 65^o Celsius. The lower melting temperature of the primer may give unspecific amplification in the PCR results. Primers with melting temperature more than 65^o Celsius have the potential for secondary annealing (Abd-Elsalam. 2003). Primer to be designed must have reasonable GC content, i.e. 55-65%. In general, the length of the PCR product has an impact on the efficiency of amplification. In this experiment, amplicon sizes ranged between 80-220bp to ensure discrimination of desired product in the qPCR results with a lower elongation time. A short amplicon < 80bp in SYBR green assays may give false Ct value in the experiment (S Bustin et al. 2017). The annealing temperature of both primers must be close to ensure their binding and amplification of specific sequence. Lastly, for each primer sequence, BLAST has been performed on the whole-genome sequence of each strain. All the primers showed positive

binding to ensure the amplification of targeted fragment on the whole genomes of selected *Streptomyces* strains.

4.2 Wet lab optimization of selected primer pairs and strain tracking

After optimization, six sets of primers were selected as strain-specific primers — one each for strain EN16 and CP200B, two each for HCA1273 and BD141. Specific primers for *Streptomyces* strains gave positive amplification on their own genome and negative amplification on closely related genomes. After getting these results, it can be considered to detect the targeted *Streptomyces* strains in the soil, roots and shoot using these strain-specific primers. Recent research revealed that optimization of primers using different strategies could be a beneficial technique to target specific microbial populations (Cannon et al. 2019; Sambo et al. 2018).

In this experiment, the qPCR assay was developed to detect and quantify the three *Streptomyces* strains in the root, shoot and soil using strain specific optimised primers. The qPCR assay was designed to ensure the quality amplification of a specific targeted fragment of a bacterial strain.

A standard curve was created using a serial dilution from the pure culture DNA of three *Streptomyces* strains, i.e. CP200B, EN16 and HCA1273 to compare the amount of DNA present in the plant samples to be quantified. A standard curve is generally used to determine the efficiency of amplification. It represents the linear regression of a plot of Ct (y-axis) vs log (DNA quantity) (S A J M Bustin. 2010). R^2 value on standard curve greater than 0.98 represents the good confidence in correlation for Ct value and DNA concentration (Bustin et al. 2017).

At the growth period of week 4, high levels of *Streptomyces* strains CP200B and EN16 was detected in the rhizosphere and roots respectively. In chickpea, recognition of CP200B in root and shoot increased at week 8. Other bacteria tend to colonise at early stage of growth while *Streptomyces* and then fungi dominate at mid or late growth period (Chauhan et al. 2012).

Similarly, in this experiment, at week 8 (mid growth stage) chickpea revealed the abundance of CP200B in root, shoot and soil. Similarly, the glass house trail with CP200B showed the better growth of chickpea plant at week 8, i.e. total biomass, nodulation. In wheat, colonization of *Streptomyces* was recorded more at week 4 and increased until week 12 (Araujo et al. 2017; Franco et al. 2007). After that colonization tends to decrease with reduction in total green mass of plant. Furthermore, root endophytic bacteria in wheat is sensitive to soil moisture. Similarly, results from this experiment depicted the reduction in EN16 strain at week 20 in wheat. Low presence of moisture in plant and rhizosphere could be the one reason of reduction in colonization. Competition with other bacterial populations could be the reason for no further colonization in roots and soil (Bonaldi et al. 2015; Compant et al. 2005).

Seed inoculation with *Streptomyces* strain CP200B had increased the colonization of the strain in the shoot and rhizosphere along with growth of the plant. In the rhizosphere and shoot, a lower concentration was detected at week 4 then increased at week 8. Higher colonization of this endophyte could be lead to changes in diversity due to competition with other bacterial populations to enhance the plant growth. The release of a large amount of antibiotics in rhizosphere might change the microbial population dynamics as well as selection of resistance. Concentration of the antibiotics in contact with the pathogens in the rhizosphere is key to the development of resistance (Olanrewaju et al. 2019).

Streptomyces strain EN16 was detected in the soil in non-diseased and *Rhizoctonia* affected wheat plants at week 4 and week 20. In the rhizosphere of the non-diseased plant a higher amount of EN16 was detected at week 4, which decreased at week 20. Antibacterial and antifungal properties of *Streptomyces* could be helpful to reduce the colonization of rhizoctonia in many plants (Sabaratnam et al. 2002). In this result, there was no rhizoctonia seen in the presence of strain EN16 at week 4 and there was a very low amount of strain EN16 detected in rhizoctonia affected plant at week 4. In rhizoctonia affected plants, the level of strain EN16

had increased at week 20. It can be concluded that early growth presence of EN16 in the soil could be helpful to restrict rhizoctonia. The presence of *Streptomyces* in could be used to control the colonization of rhizoctonia in soil (Errakhi et al. 2016; Khendkar et al. 2018)

Streptomyces strain HCA1273 was quantified in the roots and soil sample of wheat at week 4, 8 and 20. As a positive control, quantification was observed in the seed sample coated with *Streptomyces* strain HCA1273. No detection was observed in all of the soil samples. A minimal amount of HCA1273 was observed in the root samples. The presence of inhibitors in the DNA samples could be the one possibility to get negative results. PCR inhibitors can be introduced at any step prior to amplification of sample, i.e. extraction, handling, storage and many more (Schrader et al. 2012). Potential sources for inhibition include humic and tannic acids in soil (Mccord et al. 2015). There was a possibility of exclusion or loss of DNA in the extraction process. Generally, most of the recovered DNA from the samples is not proportional to the initial amount of inoculated biocontrol agent.

Some non-specific amplification was detected in the control root samples from the plant grown under application of selected three strains. These three strains were previously isolated from the the roots. So, there are chances to amplify the same strain in the DNA from the root samples. Diversity of microbial population in the roots is high than other plants tissues (Compant et al. 2010; Rosenblueth et al. 2006). There are more chances for a primer to amplify non specific product in DNA sample with high diversity in qPCR assay. Better optimization of primers, PCR and qPCR assay could be helpful to track a specific bacterial strain.

4.3 Conclusion

The experiment was established to design specific markers based on the biosynthetic genes to track the presence of *Streptomyces* strains in rhizosphere and plant. Out of 22 designed primers, six primers were considered as strain specific. Primers for CP200B and HCA1273 showed

positive quantification results in qPCR to track strains in the soil, root and shoot in chickpea and wheat. In Chickpea, strain CP200B showed more abundance in shoot, root and soil until week 8. *Streptomyces* strain EN16 revealed a lower presence of the strain in rhizosphere soils at week 20 as compared to the rhizosphere soils collected at week 4. Some non-specific amplification was seen in the root samples.

In future, designing more strain specific primers for one strain could be helpful to ensure the successful tracking of bacterial strain in the roots. The finding of unique gene copies not shared by majority of the *Streptomyces* strains with the application of PAN genome could be beneficial tool to design markers and track any specific strain.

APPENDICES

Appendix 1: Growth media and buffers

1: ISP2 Medium

Yeast extract	4g
Malt extract	10g
Dextrose	4g
Agar	20g
Distilled water	1000 ml

pH 7.2

2: Modified CTAB Method

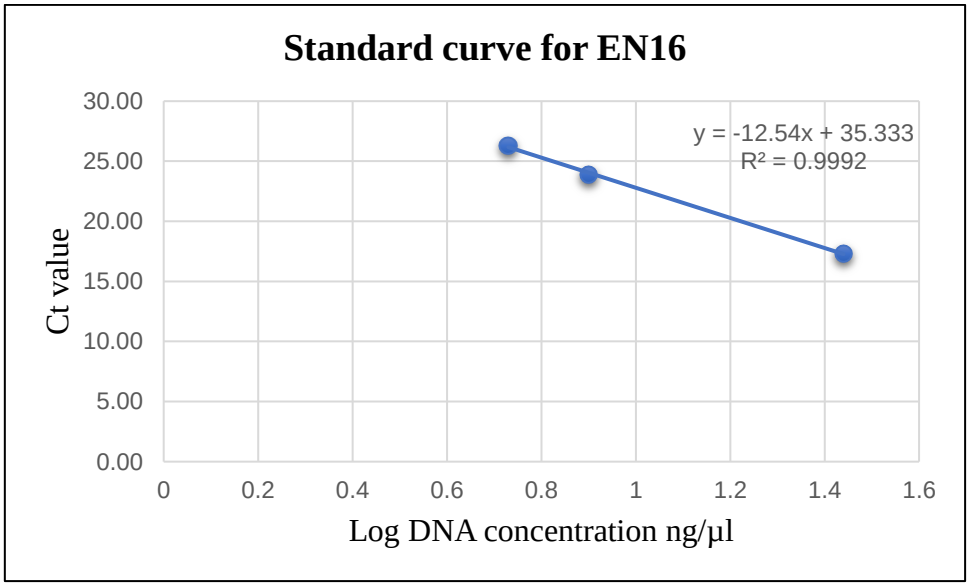
Modified CTAB buffer (0.7M NaCl)	4.9g/100ml 10% CTAB
244 mM potassium phosphate buffer	4.180g/100ml water
PEG/NaCl solution	9.93g/100ml and add 30 g PGE (6000)

3: TBE buffer (5x)

Tris base	54 g
boric acid	27.5g
0.5 M EDTA solution	20 ml
Deionized water	up to 1000ml

Appendix 2: Log DNA concentration ng/µl and Ct value of standard dilutions for *Streptomyces* strain EN16

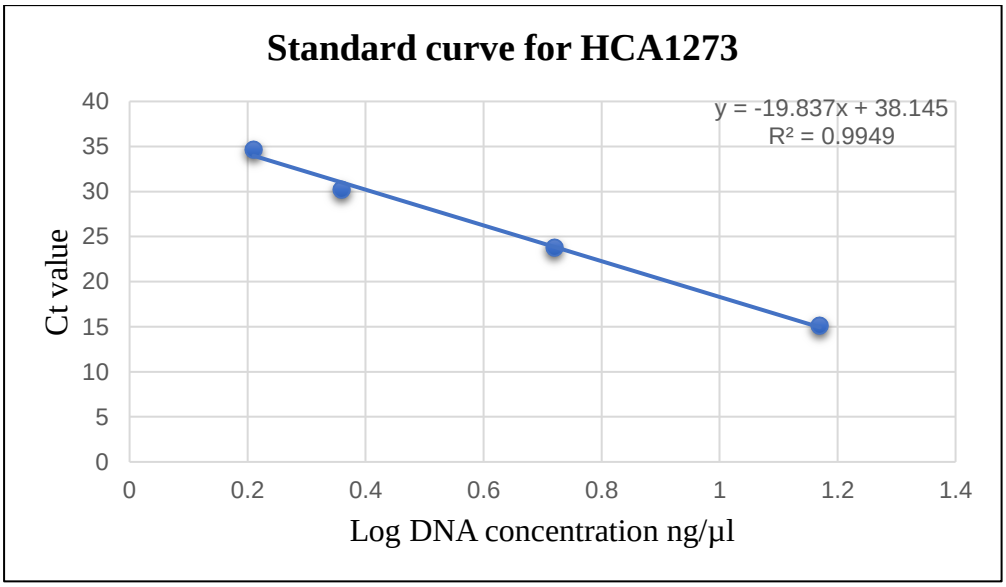
DNA Dilution	Log10 DNA amount (ng/µl)	Ct value
Dilution 1	0.99	17.31
Dilution 2	0.67	23.90
Dilution 3	0.398	26.29



Appendix 3: Standard curve plotted with Ct value and Log DNA concentration from a serial dilution of EN16 pure culture DNA**.**

Appendix 4: Log DNA concentration ng/µl and Ct value of standard dilutions for *Streptomyces* strain HCA1273

DNA Dilution	Log10 DNA amount (ng)	Ct value
Dilution 1	1.17	15.1
Dilution 2	0.72	23.78
Dilution 3	0.36	30.23
Dilution 4	0.21	34.67



Appendix 5: Standard curve plotted with Ct value and Log DNA concentration in strain HCA1237 serial dilutions.

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