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The effect of rhizobacterial volatile organic compounds on growth, modulation of gene expression, and *Agrobacterium tumefaciens*-mediated genetic transformation in *Solanum tuberosum*

Thesis presented by

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for the degree of

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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"*We choose to go to the Moon in this decade and do the other things, not because they are easy, but because they are hard*"

Abbreviations

ABA	Absicic Acid
ACC	1-Aminocyclopropane-1-carboxylic acid
AMF	Arbuscular Mycorrhizal Fungi
AMT	*Agrobacterium tumefaciens*-mediated transformation
ANOVA	Analysis of Variance
BVC	Bacterial Volatile Compound
CRISPR	Clustered Regularly Interspaced Short Palindromic-Repeats
DEG	Differentially Expressed Gene
DMDS	Dimethyl Disulfide
DVB	Divinylbenzene
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene
GC/MS	Gas Chromotography Mass Spectrometry
GM	Genetic Modification
GUS	β-glucuronidase
HCL	Hydrochloric Acid
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethylammonium bromide
IAA	Indole Acetic Acid
ISR	Induced Systemic Resistance
JA	Jasmonic Acid

LB	Luria Broth
MACE	Massive Analysis of cDNA Ends
M+S	Murashige and Skoog
MR-VP	Methyl Red Voges Proskeur
mVOC	Microbial Volatile Organic Compound
NAD	Nicotinamide Adenine Dinucleotide
NB	Nutrient Broth
NBG	Nutrient Broth with Glucose
NHR	Non-Host Resistance
NPA	Naphthalic Acid
NPRIB	National Botanical Research Institute Phosphate
O-CAS	Overlay Chrome Azurol S
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDMS	Polydimethylsiloxane
PE	Polyurethane
PGP	Plant Growth-Promotion
PGPF	Plant Growth Promoting Fungi
PGPM	Plant Growth-Promoting Microbes
PGPR	Plant Growth-Promoting Rhizobacteria
PGSC	Potato Genome Sequencing Consortium
PIPES	1,4-Piperazinediethanesulfonic acid sodium salt
PPP	Plant Protection Products

PR	Pathogenesis-Related
PTR/MS	Proton-transfer-reaction mass spectrometry
QS	Quorum Sensing
QQ	Quroum Quenching
Rsc	*Ralstonia solanacearum*
SA	Salicylic Acid
SPME	Solid Phase Microextraction
SPSS	Statistical Package for the Social Sciences
TCA	Tricarboxylic Acid
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VOC	Volatile Organic Compound
ZSB	Zinc Solubilising Bacteria

Abstract

The need for broad-ranging sustainable solutions in agriculture are needed now more than ever and potato, the third most important food crop in the world, represents an ideal crop plant in which to test these solutions. An isolation campaign from potato soils in both organic and conventional crops across the Southern coast of Ireland resulted in the identification of 21 isolates which were biochemically screened in vitro for plant growth-promoting (PGP) activities. Among these activities, the production of volatile organic compounds was of particular focus, the constituents of volatile blends from five of these native Irish potato soil isolates from the genera *Bacillus*, *Pseudomonas* and *Serratia* were identified using GC/MS, along with a commercial *Bacillus* isolate. The effect of these volatiles on plant growth was investigated using a novel experimental setup for plant-bacterial co-cultivation through the use of Microbox® growth chambers. Plant growth promotion was observed after exposure to all isolates volatile blends and varied depending on isolate and microbial growth medium, the strongest effect was observed from *Pseudomonas* and *Bacillus* isolates. Transcriptomic analysis in plants treated with *Bacillus* and *Serratia* volatiles revealed the upregulation of genes associated with photosynthesis and immune attenuation, and down regulation of a suite of defence genes. Genetic transformation mediated by *Agrobacterium tumefaciens* was also increased as a result of this growth system. This study has highlighted that volatile-mediated interactions between bacteria and plants can be

used across organic, conventional and biotechnological settings to improve food security and further our understanding of the role of bacteria in sustainable food production.

Thesis Layout

Chapter 1 examines, through an extensive literature search, the use of plant growth-promoting microbes to fortify various crop plants with micronutrients such as selenium, iron and zinc through a process designated 'biofortification' and addresses their potential implications for human health, especially in the developing world, where these microbes can play a major part in sustainable agriculture. I contributed to the majority of the scholarly work in this chapter.

Chapter 2 examines the literature surrounding the importance of microbial, and in particular, rhizobacterial volatile organic compounds for sustainable agricultural applications through determining their effect on both phytopathogen, host and ecosystems biology. These compounds will likely play significant roles in a second green revolution and can act as leads for new plant protection products which could replace more ecologically harmful synthetic alternatives. I contributed to the majority of the scholarly work in this chapter.

Chapter 3 describes the isolation, in vitro biochemical characterisation and identification of 21 potato soil bacterial isolates obtained from both organic and conventional potato crop sites across the Southern coast of Ireland. Biochemical tests carried out for plant growth-promoting traits were the production of ammonia, hydrogen cyanide and siderophores, along with solubilisation of tricalcium phosphate and emission of volatile organic compounds with growth-inhibiting activity against *Rhizoctonia solani*. Identification of soil bacterial isolates was carried

out using Sanger sequencing-based 16S rRNA. I carried out the majority of the practical and scholarly work for this chapter apart from 16S rRNA sequencing.

Chapter 4 describes the effect of rhizobacterial volatile organic compounds on plant growth-promotion and gene expression in *Solanum tuberosum* L. cv. Golden Wonder in a novel experimental setup using a passively-ventilated growth chamber. Differential gene expression is analysed post-volatile exposure to volatiles from an Irish potato soil *Serratia* isolate and a commercial *Bacillus* isolate using massive analysis of cDNA ends (MACE), a variation of traditional RNA-seq. Qualitative determination of isolate volatile blends is carried out using solid phase microextraction GC/MS. I carried out the majority of the scholarly and practical work for this chapter at UCC including the experimental design and execution of the plant growth experiments, extraction of plant RNA and the generation of GC/MS data. The commercial company, GenXPro, carried out MACE RNA-seq. The ixon-pathogenic analysis was carried out by Dr. Simone Coughlan at NUIG.

Chapter 5 describes the effect of rhizobacterial volatiles from an Irish soil *Serratia* isolate and a commercial *Bacillus* isolate cultured on two different media types on the efficiency of *Agrobacterium tumefaciens* mediated transformation in *Solanum tuberosum* L. cv. Golden Wonder by the strains AGL1 and EHA105 and provides leads based on volatile organic compounds which may be used as pre-treatments to aid improvement of transformation rates in future plant transformation

studies. I carried out the majority of the scholarly and practical work for this chapter at UCC.

General Thesis Introduction

The exponential growth of the human race, now numbering over seven billion is driving the need for increased food production and coupled with this, the need for greater food security. The need for food drives the intensity of agricultural processes ever higher and in turn damages local and global ecosystems, ultimately leading to an unsustainable and climate-damaging effect caused by modern agricultural practices. Indeed, our intensive production of food appears to already be creating a negative feedback loop in terms of food security with the observation that food production may actually be decreasing in certain parts of the planet as a result of climate change (Ray et al. 2019).

A warming climate harbouring a hungry population poses major problems for our food security, to mitigate the effects of our activities on the global ecosystem we need to find a sustainable solution to these problems. Major challenges facing agriculture include those seen in parts of the globe such as the European Union, where the use of synthetic plant protection products (PPPs) is becoming ever more restrictive and climates are changing at pace, often in favour of phytopathogens and pests which can further reduce crop yields. In poorer parts of the globe, the effects are stark. Agriculture can often be negatively affected due to drought, flooding, war or through farmers not being able to afford the basic PPPs needed to get the crop to harvest (Clair and Lynch, 2010; Gleick, 2014). People in these areas of the world are often nutrient deficient as a result of food insecurity (Daly et al. 2017).

The relationship between crop plants and the beneficial microbes which live in and around these plants offers a largely, and until recently, untapped resource for more sustainable food production and ultimately greater food security. Rhizobacteria are those which live within the immediate area of the plant host's root system and often promote growth of the host, these bacteria are termed 'plant growth-promoting rhizobacteria' (PGPR). These PGPR often form a symbiosis with the host, where the host provides a rich carbon source for bacterial nutrition through root exudates which leach into the soil of the rhizosphere, PGPR in turn help to keep the host plant healthy through a number of plant-growth promoting (PGP) activities.

These activities are broad in both their scope and effect and can influence PGP in either a direct or indirect manner. Direct PGP activities include those such as phosphate solubilisation and sequestration of iron via siderophore production. Phosphate solubilisation activity is an attractive PGP trait among PGPR candidates as phosphorus (P) is both an essential plant elemental nutrient and, crucially, a finite resource (Geissler et al. 2019). Due to this limiting factor, P-inputs are often unaffordable in the quantities needed for farmers in developing nations. A solution to this problem is the application of PGPR to agricultural systems which can increase the amount of available P for uptake in crops through increased solubilisation of P, leading to cheaper and more sustainable resource allocation in both developing and developed nations (Adnan et al.

2020). This can also be of benefit to the environment as eutrophication of waterways via field run-off can be reduced (Susser et al. 2020).

Iron is a crucial element for life, it acts as a co-factor for enzymes involved in many metabolic reactions and is converted into its biologically available form in the mitochondrion by the iron-sulfur cluster and heme synthesis pathways (Levi and Rovida, 2009). Siderophores are organic compounds with low molecular masses and their primary function is to chelate ferric iron, these compunds can be secreted by many PGPR and can sequester iron from the bulk soil which may otherwise be unavailable for uptake by bacteria. Plants can also take up the chelated iron in bacterial siderophore complexes and utilise this iron for many cellular and metabolic processes (Ahmed and Holmström 2014; Grobelak and Hiller, 2017). Another beneficial application for this interaction is the production of biofortified crops rich in iron which can address health problems faced by many developing nations whose populations are largely iron-deficient (Daly et al. 2017). Indirect PGP activities can include those related to ammonia production, hydrogen cyanide (HCN) production and the emission of volatile organic compounds (VOCs), all these activities have implications for microbial ecological competition and plant growth respectively (Howell et al. 1988; Ryu et al. 2003; Rijavec and Lapanje, 2016; Anand et al. 2020).

In nature, the vast majority of many terrestrial organisms communicate through the use of VOCs. These compounds can be used for example by mammals when finding a mate or marking territory (Dulac and

Torello, 2003; Marneweck et al. 2017), by insects such as ants for maintaining food supplies from fungus gardens (Green and Kooij, 2018), by plants to indicate to their surrounding plant neighbours that they are under herbivorous attack (Arimura et al. 2009) or by bacteria to inhibit the growth of phytopathogens (Asari et al. 2016). The VOCs produced by bacteria can be referred to as bacterial volatile compounds (BVCs) (Chung et al. 2016). These BVCs form part of a wider communication network between microbes across different ecological settings (Schmidt et al. 2019) and can be utilised by PGPR to protect their respective host plants from attack by phytopathogens by (Sharifi and Ryu, 2018a). Plants recognise these BVCs and modulate both their genomic and physiological architecture to prepare for any potential attack should the direct PGPR defence be circumvented by the phytopathogen, this phenomenon is termed induced systemic resistance (ISR) (Ryu et al. 2004; Farag et al. 2013). The induction of ISR is mediated by alterations in gene expression in the plant after perception of either the single or blend of volatiles, these genes are often involved in the defence and/or stress response in plants (Shi et al. 2017; Sharifi and Ryu, 2018b).

The soil-borne plant pathogen *Agrobacterium tumefaciens* is often referred to as a natural ‘genetic engineer’ owing to its particular life strategy of infecting a plant host and creating a specialised tumour or ‘crown gall’ within the plant where, via a tumour-inducing (Ti) plasmid which is a mobile genetic element, it can ‘hijack’ the genome of the plant cell to make the tumour and subsequently produce nutrients for

continued bacterial growth within the tumour (Binns and Thomashow, 1988; Hwang et al. 2017). This process can often be seen in nature by the presence of 'crown gall' disease of many plant species (Matveeva and Otten, 2019).

To achieve this successful infection and colonisation of its host, *A. tumefaciens* employs an arsenal of intracellular effectors which try to both evade and hijack the plant hosts innate immune response to allow genomic integration of the transfer DNA (T-DNA) (Pitzschke, 2013). Since BVCs are known to alter gene expression in sensor plants, can we capitalise on the effects of this response to increase *Agrobacterium tumefaciens*-mediated transformation (AMT) efficiency in potato? The potential for PGPR to contribute to all agricultural regimes and biotechnological applications can be a significant boon in our attempt to feed the world's population while further still, lessening our negative impact on the climate and ecosystems of Earth.

The aims of this body of work are; 1) to investigate to potential of PGPR for sustainable agriculture, with particular focus on the utilisation of volatile organic compounds. 2) to isolate potential PGPR from both organic and conventional potato crops in Ireland, characterise their respective biochemical PGP activities and identify these PGPR through 16S rRNA analysis. 3) to determine PGP effects of BVCs emitted from these bacterial isolates in a novel experimental system devised for BVC-mediated plant growth in potato microplants followed by transcriptomic analysis in response to a number of these

respective bacterial BVCs. 4) to determine the effect, if any, of BVC pre-treatment of whole potato microplants to AMT in potato.

References:

Adnan M, Fahad S, Zamin M, Shah S, Mian IA, Danish S, Zafar-ul-Hye M, Battaglia ML, Naz RM, Saeed B, Saud S (2020) Coupling phosphate-solubilizing bacteria with phosphorus supplements improve maize phosphorus acquisition and growth under lime induced salinity stress. Plants 9:900

Ahmed E, Holmström SJ (2014) Siderophores in environmental research: roles and applications. Microb biotechnol 7:196-208.

Anand A, Chinchilla D, Tan C, Mène-Saffrané L, L'Haridon F, Weisskopf L (2020) Contribution of hydrogen cyanide to the antagonistic activity of *Pseudomonas* strains against *Phytophthora infestans*. Microorganisms 8:1144

Arimura GI, Matsui K, Takabayashi J (2009) Chemical and molecular ecology of herbivore-induced plant volatiles: proximate factors and their ultimate functions. Plant Cell Physiol 50:911-923

Asari S, Matzén S, Petersen MA, Bejai S, Meijer J (2016) Multiple effects of *Bacillus amyloliquefaciens* volatile compounds: plant growth promotion and growth inhibition of phytopathogens. FEMS Microbiol Ecol 92:fiw070

Binns AN, Thomashow MF (1988) Cell biology of *Agrobacterium* infection and transformation of plants. Ann Rev Microbiol 42:575-606

Chung JH, Song GC, Ryu CM (2016) Sweet scents from good bacteria: case studies on bacterial volatile compounds for plant growth and immunity. Plant Mol Biol 90:677-687

Clair SB, Lynch JP (2010) The opening of Pandora's Box: climate change impacts on soil fertility and crop nutrition in developing countries. Plant Soil 335:101-115

Daly DH, Velivelli SLS, Prestwich BD (2017) The Role of Soil Microbes in Crop Biofortification. In: Meena V, Mishra P, Bisht J, Pattanayak A (eds) Agriculturally Important Microbes for Sustainable Agriculture. Springer, Singapore, pp. 333-356

Dulac C, Torello AT (2003) Molecular detection of pheromone signals in mammals: from genes to behaviour. Nat Rev Neurosci 4:551-562

Farag MA, Zhang H, Ryu CM (2013) Dynamic chemical communication between plants and bacteria through airborne signals: induced resistance by bacterial volatiles. J Chem Ecol 39:1007-1018

Geissler B, Mew MC, Steiner G (2019) Phosphate supply security for importing countries: Developments and the current situation. Sci Total Environ 677:511-523

Gleick PH (2014) Water, drought, climate change, and conflict in Syria. Weather Clim Soc 6:331-340

Green PW, Kooij PW (2018) The role of chemical signalling in maintenance of the fungus garden by leaf-cutting ants. Chemoecology 28:101-117

Grobelak A, Hiller J (2017) Bacterial siderophores promote plant growth: screening of catechol and hydroxamate siderophores. Int J Phytoremediation 19:825-833

Howell CR, Beier RC, Stipanovic RD (1988) Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. Phytopathology 78:1075-1078

Hwang HH, Yu M, Lai EM (2017) *Agrobacterium*-mediated plant transformation: biology and applications. The Arabidopsis Book 15

Levi S, Rovida E (2009) The role of iron in mitochondrial function. Biochim Biophys Acta (BBA)-General Subjects 1790:629-636

Marneweck C, Jürgens A, Shrader AM (2017) Dung odours signal sex, age, territorial and oestrous state in white rhinos. Proc Royal Soc B: Biological Sciences 284:20162376

Matveeva TV, Otten L (2019) Widespread occurrence of natural genetic transformation of plants by *Agrobacterium*. Plant Mol Biol 101:415-437

Pitzschke A (2013) *Agrobacterium* infection and plant defense—transformation success hangs by a thread. Front Plant Sci 4:519

Ray DK, West PC, Clark M, Gerber JS, Prishchepov AV, Chatterjee S (2019) Climate change has likely already affected global food production. PloS one 14:e0217148

Rijavec T, Lapanje A (2016) Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. Front Microbiol 7:1785

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017-1026

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Paré PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci U. S. A. 100:4927-4932

Schmidt R, Ulanova D, Wick LY, Bode HB, Garbeva P (2019) Microbe-driven chemical ecology: past, present and future. ISME J 13:2656-2663

Sharifi R, Ryu CM (2018a) Revisiting bacterial volatile-mediated plant growth promotion: lessons from the past and objectives for the future. Ann Bot 122:349-358

Sharifi R, Ryu CM (2018b) Sniffing bacterial volatile compounds for healthier plants. Curr Op Plant Biol 44:88-97

Shi Y, Niu K, Huang B, Liu W, Ma H (2017) Transcriptional responses of creeping bentgrass to 2,3-butanediol, a bacterial volatile compound (BVC) analogue. Molecules 22:1318

Susser JR, Pelini SL, Weintraub MN (2020) Can we reduce phosphorus runoff from agricultural fields by stimulating soil biota? J Environ 49: 933– 944

Chapter 1

The role of soil microbes in crop biofortification

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Abstract:

Agronomic practices across the planet are becoming largely unsustainable in their current forms. With a growing population expected to reach ~9 billion by the year 2050, more sustainable ways to produce the world's main crops are needed. The main focus of current agronomic practices, especially in the case of cereal crops, is increased grain number and weight sometimes at the expense of nutritional content leading, in some instances, to micronutrient deficiencies. Micronutrient deficiencies are often termed '*hidden hunger'*, giving the false appearance that an individual is consuming sufficient amounts of nutrients. To counteract this problem, it is crucial that a sustainable solution to increase micronutrient concentration in a diverse range of crops is found. Plant growth- promoting microbes

(PGPM) represents a sustainable solution to this problem. These PGPM can be divided into two main groups: plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF). These microbes are capable of increasing micronutrient concentrations in many crops worldwide. This chapter will focus on the use of these microbes to increase micronutrient content, in particular selenium, iron and zinc, using studies conducted over the last two decades right up to the present day, revealing how plant-microbe interactions and our ever-growing knowledge of these interactions can aid in the micronutrient biofortification of crops in a sustainable and environmentally friendly way.

Introduction:

The pressure to produce sufficient nutritionally rich food in a sustainable manner has never been greater. With a growing global population (~9 billion by 2050), changing demographics and climate, pressure on resources such as water and land use, emergence of novel and more virulent pests and pathogens and changes in regulations with respect to the use of agrichemicals, food security is predicted to become increasingly vulnerable (FAO 2016). The United Nations through the 'Sustainable Development Goals Project' (UN 2015) has identified 17 goals to transform our world. Second on this list is the goal to 'end hunger, achieve food security and improve nutrition and promote sustainable agriculture'.

Nowadays, ~800 million people are undernourished with the vast majority living in developing countries. It is estimated that malnutrition contributes to the death of ~3.0 million children annually. Micronutrient deficiencies also known as hidden hunger where the intake of various vitamins and minerals (e.g. iron, iodine, selenium, zinc, folic acid and vitamin A) is too low for optimal health are a global issue affecting ~2 billion people (Trijatmiko et al. 2016). Nutritional deficiencies not only impact on an individual's health but also on their economic prosperity. The economic cost of micro- and macronutrient deficiencies has been estimated at ~2 trillion US dollars annually. Therefore, improving nutritional health is probably the major socio-economic challenge of this century. The nutritional content of our food crops can be enhanced in various ways. This enhancement is termed 'biofortification' which according to the World Health Organization (WHO) is defined as 'the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology' (WHO 2016a).

Probably the most famous biofortification project using genetic modification is the production of Golden Rice (Golden Rice 2016), whose benefits although clearly identified has faced much public opposition where it is perceived by many as the 'Trojan horse' for genetically modified crops. Genetic modification, particularly in Europe, is anathema to many, and as a consequence, Golden Rice is still not commercially available (International Rice Research Institute 2016). Besides genetic transformation approaches to biofortification,

other biofortification strategies, including initiatives such as those followed in programs by HarvestPlus (2016) for the biofortification of rice, involve a combination of conventional breeding strategies for the exploitation of existing genetic variation in crop germplasm coupled with agronomic strategies, e.g. foliar and soil application of fertilizers (Nakandalage et al. 2016; Amanullah et al. 2016; Meena et al. 2013a; Bahadur et al. 2014; Maurya et al. 2014; Kumar et al. 2016c).

More recently, an understanding of the complex interactions between plant roots and microbial communities in the rhizosphere has fuelled research into the role and application of soil microbes in the biofortification of crops. There are many reports in the literature on the role of bacteria and mycorrhizal fungi in the mobilization of micronutrients in plants (Wu et al. 2015; Wang et al. 2014; Krishnakumar et al. 2013; Berruti et al. 2016). This chapter will focus in particular on crop biofortification by bacterial- and mycorrhizal-mediated mobilization of the micronutrients iron (Fe), zinc (Zn) and selenium (Se) from the rhizosphere (Jat et al. 2015; Kumar et al. 2015, 2016b; Ahmad et al. 2016; Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Jaiswal et al. 2016; Jha and Subramanian 2016).

The Application of Plant Growth-Promoting Rhizobacteria for the Biofortification of Crops:

The rhizosphere, an important interface between plant roots and the soil, can contribute to sustainable agriculture when the interaction between plants and beneficial bacteria is exploited. It has been ~35 years since Kloepper first described the role of plant growth-promoting rhizobacteria (PGPR) in plant growth and defence (Kloepper et al. 1980). PGPR when associated with rhizosphere/plant roots play a major role in the direct or indirect promotion of plant growth. The direct plant growth promoter mechanisms, biofertilization and phytostimulation, are two examples of methods that simultaneously minimize the use of chemical fertilizers and promote plant growth, and bacteria with both biocontrol and biofertilization/phytostimulation properties offer advantage to plants in terms of both enhanced nutrient supply and control of disease (Adesemoye and Kloepper 2009; Lugtenberg and Kamilova 2009; Glick 2012; Velivelli et al. 2014).

The recent work in the area of plant-microbe interactions has focused on the biofortification of staple crops using these PGPR, showing significant results. The WHO has identified micronutrients which are essential to the correct functioning of the human body, i.e. selenium (Se), iron (Fe) and zinc (Zn), and these constitute a significant portion of the current research on PGPR-mediated biofortification (Priyadharsini and Muthukumar 2016; Kumar et al. 2017; Meena et al. 2015a, b, f; Raghavendra et al. 2016; Zahedi 2016; Dominguez-Nunez

et al. 2016; Dotaniya et al. 2016). These three micronutrients will be discussed further in relation to the biofortification of crops using PGPR.

***Selenium*:**

The Importance of the Trace Element Selenium in the Human Diet:

Selenium (Se), a significant trace element in the human diet, is classified as a metalloid and occurs in the environment in different valences as selenide (^{2}-), selenite ($^{4+}$), selenate ($^{6+}$) or elemental Se (0). Se has implications for biological processes, such as anti-oxidation and prevention of certain cancers, e.g. liver cancer (Carlson et al. 2012) and colorectal cancer (Meplan and Hesketh 2012). Selenoproteins, i.e. proteins that contain a selenocysteine amino acid residue, facilitate adequate DNA synthesis, protection against diseases such as HIV, reproduction and thyroid hormone metabolism (Sunde 2012; Hatfield et al. 2014). A delicate balance occurs in humans between having too much and too little Se in the diet. Se levels that are too high can lead to selenotoxicity, resulting in symptoms ranging from nervous system disorders to nail and hair loss (Li et al. 2012), and selenium levels that are too low can lead to inadequate levels of protection against radioactive damage to DNA, which can result in micronuclei formation and potentially cancer (Baliga et al. 2008).

The Role of PGPR in the Mobilization of Selenium for the Biofortification of Plants:

Plant roots have the capacity to absorb Se as selenate, selenite or organo-selenium compounds such as selenocysteine and selenomethionine, but lack the ability to absorb metal selenides or elemental Se (White et al. 2004, 2007). Generally, Se concentrations found in plants grown in seleniferous soils are less than 25 mg kg^{-1} dry weight (Bell et al. 1992; Terry et al. 2000; Yasin et al. 2015a, b), but in the case of a few hyperaccumulator species, these values can reach over 1000 mg kg^{-1} dry weight in plant material (Ellis and Salt 2003).

Selenate is the most widely utilized form of Se by plants, and in non-hyperaccumulators selenate is transported across the plasma membrane of root cells by high-affinity sulphate transporters (Terry et al. 2000; White et al. 2004, 2007; Sors et al. 2005; Broadley et al. 2006; Hawkesford and Zhao 2007). Plant growth- promoting rhizobacteria (PGPR) have the potential to increase plant Se levels and subsequently benefit the Se status of humans and livestock in areas of the world which may be Se-deficient. Over the past two decades, a number of studies have examined the potential of PGPR to act as Se-biofortification agents. A study by de Souza et al. (1999) found that Indian mustard (*Brassica juncea* L.) can be supported by PGPR to enhance selenium accumulation and volatilization (Rawat et al. 2016; Yasin et al. 2016; Meena et al. 2016c, d; Saha et al. 2016b; Yadav and Sidhu 2016; Bahadur et al. 2016b; Das and Pradhan 2016).

In *B. juncea*, dimethyl selenide is the predominant volatilized form of Se and is typically 500–700 times less toxic than inorganic forms of Se (McConnell and Portman 1952; Ganther et al. 1966; Wilber 1980) making it a suitable vector for biofortification of the human food chain with Se. A number of Se-tolerant rhizobacterial isolates from the rhizosphere of a range of Se-accumulating plants were used in this study. Antibiotic experiments using ampicillin revealed that bacteria were the key factor in enhanced Se uptake by Indian mustard (de Souza et al. 1999). When added to the plant nutrient solution, ampicillin inhibited Se volatilization by ~35% and tissue Se accumulation by ~70%.

The rhizobacterial strains designated BJ2 and BJ15 were used in axenic plant experiments to examine the role of rhizobacteria in Se accumulation and volatilization. When these two strains were inoculated within the rhizosphere of axenic Indian mustard plants, the Se volatilization rate from selenite was four times higher than that of axenic control plants, and plants accumulated more Se in their tissues when compared to axenic controls. Both BJ2 and BJ15 rhizobacterial strains increased Se concentrations in shoots and roots 1.4-fold and fivefold, respectively. As with many PGPR species, these isolates were thought to increase Se uptake via enhanced root hair production. In addition, seedlings with PGPR had a significantly higher Se tissue concentration of 1.3–2 times greater than axenic plants across the spectrum of Se concentrations tested (de Souza et al. 1999).

Acuña et al. (2013) investigated ash-derived volcanic andisol soil in Southern Chile, which is characteristically low in Se content, for the presence of PGPR with the ability to supply increased Se to host plants. Inoculation with two isolates, namely, *Pseudomonas* sp. R8 and *Stenotrophomonas* sp. B19, in the rhizosphere of wheat resulted in higher Se content in roots and leaves compared to uninoculated controls. When plants were inoculated with B19 in soils where the Se concentration was 5–10 mM, a significantly higher Se content was observed in wheat root and leaf tissues compared to plants grown in soil containing 2 mM Se (Acuña et al. 2013). In the same region, Durán et al. (2014) isolated endophytic bacteria from Se-supplemented wheat that shows potential for plant growth promotion, biofortification and biocontrol in wheat cultivation (Saha et al. 2016c; Verma et al. 2014, 2015b; Meena et al. 2014a, 2015e; Teotia et al. 2016).

Endophytic bacterial strains were isolated from both uninoculated control and Se-supplemented wheat plants from the genera *Bacillus*, *Paenibacillus*, *Klebsiella*, *Enterobacter* and *Acinetobacter* and 15 strains selected according to tolerance of high Se levels (~50 mM). A number of these endophytes are highly tolerant to Se, ranging from 60 to 180 mM, and thus have the potential to be used as biofortification agents in areas of the world with low soil Se concentrations. Furthermore, some of these isolates displayed the ability to act as biocontrol agents for the endemic soilborne fungal pathogen *Gaeumannomyces graminis* var. *tritici* that causes take- all disease, one of the most severe wheat diseases in Southern Chile. Isolates from the

genera *Acinetobacter* (strain E6.2), *Bacillus* (strain E8.1), *Bacillus* and *Klebsiella* (strains E5 and E1) inhibited the growth of *G. graminis* var. *tritici* mycelia by ~100%, 50% and 30%, respectively, *in vitro*; this adds to the potential of PGPR for biofortification agents that not only increase Se concentration in the plant but also act as sustainable crop protectants (Durán et al. 2014).

Two studies by Yasin et al. (2015) investigated the potential for Se biofortification in crop plants. First, an experiment with *B. juncea* to investigate the potential of two Se-tolerant rhizobacterial consortia to increase plant Se uptake using naturally seleniferous soil containing ~8 mg Se kg^{-1} was carried out with both rhizobacterial consortia consisting of four to five strains each, designated G1 and G2. *B. juncea* accumulated Se to 358 mg kg^{-1} in seeds, 711 mg kg^{-1} in leaf dry weight (DW) and 276 mg kg^{-1} in pod husks. Inoculation of G1 had a positive effect on plant growth compared to controls but did not accumulate Se with a significant difference compared to the control. G1-incoulated plants produced 40–45% more leaf and seed biomass compared to controls however; therefore, these plants were more effective in extracting Se from the soil compared to control plants. G2-inoculated plants had an overall decreased plant growth compared to control plants. However, nonprotein thiol levels were increased threefold in G2-inoculated plants compared to controls. Nonprotein thiols are often used by plants as antioxidant defence compounds, and it may be the case that one or more members of the G2 consortia initiated this response to a perceived bacterial threat (Yasin et al.

2015a). In a second experiment using two individual isolates, namely, *Bacillus cereus* YAP6 and *Bacillus licheniformis* YAP7, inoculation of wheat plants with either bacteria increased Se concentration in the grain by up to 375% compared to uninoculated Se-treated plants (Yasin et al. 2015b).

As mentioned previously, plants grown in seleniferous soil that can accumulate very high tissue levels of Se are termed 'Se hyperaccumulators'. This poses the question of whether this hyperaccumulation of Se can be attributed to any specific microbe associated with Se-hyperaccumulating plants. Given the current depth of knowledge in plant-microbe interactions, we might reasonably expect such a microbe would be identified. The answer appears to be 'no'. An experiment by Sura-de Jong et al. (2015) compared the endophytic population of both non- hyperaccumulating and hyperaccumulating plants, with two plant families per grouping, Brassicaceae and Fabaceae growing on the same seleniferous site (Sharma et al. 2016; Verma et al. 2015a; Meena et al. 2013b, 2016e; Shrivastava et al. 2016).

Terminal restriction fragment length (T-RFLP) analysis of bacterial isolates identified equal T-RF diversity between hyperaccumulators *Stanleya pinnata* and *Astragalus bisulcatus* and related non-hyperaccumulators *Physaria bellii* and *Medicago sativa*. Cultivable endophytes from hyperaccumulators were isolated, and of the 66 morphotypes isolated, seven were selected to be co-cultivated with *B. juncea* and *M. sativa*. Although plant growth promotion occurred in

both plants, there was no effect on their Se status, suggesting that hyperaccumulation cannot be induced by the application of Se-tolerant PGPR alone (Sura-de Jong et al. 2015). Studies like these highlight the importance of understanding the complex interactions between all constituents of the plant-microbe interaction and how not all bacteria can benefit plant biofortification (Velazquez et al. 2016; Meena et al. 2015c; Sindhu et al. 2016; Bahadur et al. 2016a; Masood and Bano 2016) (Fig. 1).

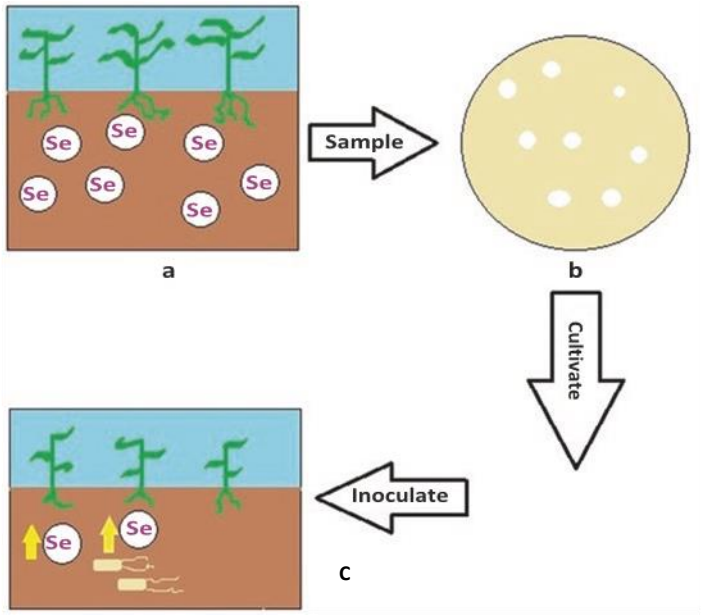


Fig. 1. Rationale for selection of PGPR involved in biofortification taking Se as an example. (**a**) Se-enriched soils. (**b**) Isolation of bacteria from (**a**) on Se-supplemented growth media to identify Se-tolerant bacteria. (**c**) Inoculation of PGPR from Se-enriched soils to Se-deficient soils to aid Se biofortification in plants.

Iron:

The Importance of Iron in the Human Diet:

Iron (Fe) is arguably one of the most important micronutrients in the human diet; it is the basis for the correct function of haemoglobin, and deficiency in iron can lead to anaemia. According to the World Health Organization (WHO), Fe deficiency is the only micronutrient deficiency that is prevalent in both the developing and developed worlds (WHO 2016b). In developing countries, up to ~50% of pregnant women and ~40% of preschool children suffer from anaemia (Beard 2008) with iron levels below those recommended WHO (Table 1). Values for recommended daily intake are displayed in Table 1. In children under 5 years of age, 0.2% of deaths can be linked to deficiency in iron (Bhutta 2008). Children suffering from anaemia and Fe deficiency are better protected against mortality or hospital admission when supplemented with iron; however, supplementation with iron to the point where children become iron replete and no longer iron deficient may be harmful as in the case of malaria, whereby the causative agent, *Plasmodium*, can benefit from excess iron levels in the blood (Sazawal et al. 2006; Weinberg 1999; Nuberger et al. 2016). So, as for all (bio)fortification strategies, the relative benefits and risks must be balanced (Meena et al. 2013c, 2014b, 2015d; Singh et al. 2015, 2016).

The Acquisition of Iron by Plants:

Plants acquire Fe via one of two strategies, depending on whether the plant is monocotyledonous or dicotyledonous. Appreciating these different mechanisms in the overall process of Fe acquisition by plants can help in the optimization strategies of Fe translocation from the rhizosphere to the plant. The reduction strategy, used by dicotyledonous plants, involves the secretion of H^+ and organic acids to acidify the rhizosphere which reduces Fe^{3+} to Fe^{2+} and makes it available for uptake into the plant. The chelation strategy, used mainly by monocotyledonous plants, involves the secretion of phytosiderophores (e.g. mugineic acid) that can bind Fe^{3+} which is subsequently taken up by the root cells (Gamalero and Glick 2011; Bulgarelli et al. 2013).

The Role of PGPR in Iron Acquisition and Plant Biofortification:

The enzymes involved in the mugineic acid pathway were deduced from sequence comparison with bacteria, as it is now well known that bacteria can also act to help plants acquire Fe (Crowley 2006; Saha et al. 2016a). In one of the early experiments to determine microbial contribution to plant Fe acquisition,

Masalha et al. (2000) experimentally determined that soil microbial activity was enhancing Fe acquisition using sterile and non-sterile soil as growth media. Non-sterile soil was associated with increased Fe uptake, suggesting that the microbial community of the soil may be contributing to Fe uptake in the plant. In sunflower (*Helianthus annuus* L.), root dry weight Fe concentrations were ~1748 ± 48 μg g^{-1} for non-sterile soil, whereas in sterile soil, Fe concentration was ~248 ± 29 μg g^{-1}, suggesting microbial activity was enhancing Fe translocation from the soil to the plant (Masalha et al. 2000). The microbial siderophores can be classified into four groups; the most common are the hydroxamate siderophores, followed by catecholates, carboxylates and pyoverdines. Examples of siderophores within these four respective groups include ferribactin, pyoverdine, enterochelin and rhizobactin; these are produced by *P. fluorescens, P. aeruginosa, E. coli* and *R. meliloti*, respectively (Maurer and Keller-Scheirlin 1968; Smith and Neilands 1984; Schalk and Guillon 2013; Vansuyt et al. 2007). Upwards of ~500 different siderophores have been reported to date, with ~270 being well characterized (Boukhalfa et al. 2003).

Siderophores can also be involved in a biocontrol capacity, sequestering iron away from the rhizospheric soil and making it inaccessible to any potential pathogens (Siddiqui 2006). These microbes can further enhance Fe acquisition in plants by inducing

Fe-deficient responses, such as the production of hormonal compounds analogous to plant hormones, which microbes can produce independently. *B. subtilis* GB03 has the ability to enhance Fe acquisition in *Arabidopsis* via transcriptional upregulation of the Fe deficiency-induced transcription factor 1 (FIT1) which is required for induction of ferric reductase FRO2 and the IRT1 iron transporter to acquire Fe in the plant (Zhang et al. 2009).

A group of major microbial-derived plant growth-promoting (PGP) hormones called auxins have been implicated in enhanced Fe uptake. The ratio of auxin- producing microorganisms in soil that contains phenolic root exudates of Fe-deficient red clover (*Trifolium pratense* L.) is higher than that observed in phenolic-free soil solution controls, thus indicating that Fe deficiency in plants may contribute to the abundance of auxin-producing microbes in the rhizosphere. Not only the increase of auxin-producing microbes was observed, but the plant phenolics also inhibited the growth of the majority of microbes, suggesting iron-deficient plants can select for organisms which have the ability to produce auxin and siderophores, ~71% and 86% of the total microbial population, respectively (Jin et al. 2006, 2008). Exogenous application of the major auxin, indole-3-acetic acid (IAA), enhances Fe deficiency-induced reduction of ferric Fe and expression of FRO2 and IRT1, which are critical for enhanced Fe

uptake in the plant and growth of lateral roots and root hairs to provide increased surface area for Fe uptake (Jin et al. 2008; Chen et al. 2010; Wu et al. 2012). IAA also contributes extra nutrients for rhizobacterial growth by modulating the quantity of root exudation via an increase of porosity in the cell wall of plants (Glick 2012).

Utilizing two of the most important cereal crops, rice (*Oryza sativa* L.) and wheat (*Triticum aestivum*) in a rice-wheat cropping system, Rana et al. (2015) demonstrated that PGPR and cyanobacteria led to both higher macro- and micronutrient concentration in cereal grain. In rice, bacterial-cyanobacterial consortia increased Fe concentration from ~4 to 14% over the fertilizer-only control. While bacterial- cyanobacterial consortia had a significant Fe increase over uninoculated controls, isolate *Providencia* sp. PW5 alone increased the Fe concentration by ~45% compared to the fertilizer-only control in wheat grains. Adak et al. (2016) also observed increased acquisition of Fe through cyanobacteria inoculation in rice of ~13 to 46% compared to uninoculated controls.

Recent publications from India on the biofortification of chickpea (*Cicer arietinum* L.), a major pulse crop grown under semiarid conditions, have highlighted the potential for Fe biofortification in chickpea. Nineteen *Acinetobacter* isolates statistically

significantly increased mineral content in PGPR-inoculated chickpeas compared to uninoculated controls; Fe in plants varied from ~10 to 38% higher (Sathya et al. 2016). All 19 isolates increased Fe in the plant within the range of ~10 to 38%. Gopalakrishnan et al. (2016) also recorded increases in Fe concentration following PGPR treatment. Fe concentration in chickpea grain was increased up to 18% with isolates *E. ludwigii* SRI-229 and *P. monteilii* SRI-360, the two strains displaying the greatest performance over uninoculated controls (Gopalakrishnan et al. 2016). It is important to note that postharvest processing and cooking can affect micronutrient concentrations in the grain. Both Saytha et al. (2016) and Gopalakrishnan et al. (2016) reported that Fe concentration can be affected by cooking and can reduce from 5 to 30% and in some cases gain ~21% (Sathya et al. 2016; Gopalakrishnan et al. 2016).

***Zinc*:**

The Importance of Zinc to Human Health:

Zinc (Zn) is typically the most abundant transition metal in living organisms after Fe and is the only metal resident in all enzyme classes (King 2006; Broadley et al. 2007). Zn plays a critical role

in human health and is involved in correct functioning of the immune system to fight infections such as pneumonia (Black et al. 2008). Zn is also involved in protein synthesis, metabolic homeostasis and modulation of gene expression through Zn-containing proteins (Welch 2001) and plays a critical role in male fertility with Zn deficiency resulting in inhibition of spermatogenesis and abnormal sperm production (Prasad 2008). In contrast to Fe, Zn is not stored in the human body in sufficient amounts and as a result must be ingested daily (King 2011). Zn, along with Fe, iodine (I) and Se, i.e. the major micronutrients, is often found in insufficient quantities in plant staple foods to meet human nutritional requirements, and as a consequence, nearly half the global population suffers from Zn micronutrient deficiency (White and Broadley 2009; Cakmak 2008) (Table 1).

The PGPR-Mediated Biofortification of Plants with Zinc:

Processing of staple grains such as rice and wheat decreases the micronutrient concentration of the grain, especially in the case of Zn in wheat (Zhang et al. 2010; Kutman et al. 2011). Grain concentration of Zn is also hindered by the presence of the well-documented ‘anti-nutrient’ phytic acid, which reduces mineral bioavailability because it can form insoluble complexes with

essential minerals such as Zn^{2+} and Fe^{3+} (Urbano et al. 2000; Srivastava 2016). Tariq et al. (2007) investigated the potential of a PGPR consortium to increase Zn concentration in rice; the consortium, named 'BioPower', consists of two *Azospirillum lipoferum* strains, two *Pseudomonas* sp. strains and one *Agrobacterium* sp. strain (Hafeez et al. 2002; Tariq et al. 2007).

Rana et al. (2012) showed that inoculation of wheat with *Providencia* sp. + $N_{60}P_{60}K_{60}$ significantly increases grain Zn accumulation to ~42 mg kg^{-1}, along with a threefold increase in the concentration of Fe compared to control plants. Zinc-solubilizing bacteria (ZSB) have also been shown to increase Zn concentration in shoots and roots of soybean and wheat (He et al. 2010). Rana et al. (2015) documented an increased Zn concentration in wheat and rice grains over a 2-year-long rice-wheat cropping sequence. In wheat grains, Zn concentration was ~41 mg kg^{-1} post-treatment with $N_{60}P_{60}K_{60}$ and a bacterial consortium consisting of *B. pumilus* PW1, *Providencia* sp. PW5 and *B. diminuta* PW7. Zinc concentration in the wheat grain was measured at ~38 mg kg^{-1} post- treatment with $N_{60}P_{60}K_{60}$ only and was measured at ~33 mg kg^{-1} for the control without the application of fertilizer or bacteria.

At the end of the 2-year cycle, Zn concentration in wheat grain had increased 1–7% overall in plants that had been treated with

both fertilizer and bacteria compared to fertilizer-only controls. In rice, Zn concentration in grain increased by ~14% when treated with $N_{90}P_{60}K_{60}$ and a PGP bacterial consortium consisting of the strains *Providencia* sp. PR3, *Brevundimonas diminuta* PR7 and *Ochrobactrum anthropi* PR10 (Rana et al. 2015).

Table 1. Recommended daily dietary intake of Se, Zn and Fe, WHO (2006)

	Se	**Zn**	**Fe**
Category	mg		
Infants (1–3 years)	17	2.4–8.3	3.9–11.6
Children (4–6 years)	22	2.9–9.6	4.2–12.6
Adult female	26	3–9.8	19.6–58.8
Adult male	34	4.2–14	9.1–27.4
Pregnant women	28	4.2–14	>50
Lactating women <3 months	35	5.8–19	10–30

Values for Zn and Fe run from low to high as bioavailability increases in the diet. A diet of low bioavailability is defined as a diet low in vitamin C and animal protein; the opposite is true for a diet with high bioavailability. Socioeconomic and dietary factors play a major role in the potential uptake of micronutrients in the human body.

Manjunath et al. (2016) recorded increased Zn concentration in okra (*Abelmoschus esculentus* (L.) Moench). During midcrop stage, Zn concentrations were ~60 to 70% higher in plants treated with *Azotobacter* sp. and a cyanobacterium (e.g. *Calothrix*), compared to un-inoculated controls (Majunath et al. 2016). Shakeel et al. (2015) observed that ZSB increased zinc translocation in two rice varieties, basmati-385 and super basmati. A consortium of *Bacillus* sp. SH-10 and *B. cereus* SH-17 yielded

a Zn concentration in grain of ~31 mg kg^{-1} compared to a concentration of ~18 mg kg^{-1} in the basmati-385 control. Application of ZSB with Zn fertilizer did not increase Zn concentration in the grain; this may be due to the increased Zn uptake potential of the varieties tested (Shakeel et al. 2015) (Table 2). Isolates hail from diverse genera based on respective abilities to fix or solubilize nutrients within the rhizosphere for plant growth promotion (Fig. 2).

Table 2. A selection of microbes which have been shown to contribute to the process of micronutrient biofortification

Microbe	Micronutrient	Reference
Bacillus subtilis (GB 03)	Fe	Zhang et al. (2009)
Pseudomonas sp. R8	Se	Acuña et al. (2013)
Stenotrophomonas sp. B19	Se	Acuña et al. (2013)
Providencia sp. PW5	Zn, Fe, Mn, Cu	Rana et al. (2012)
Pseudomonas sp. Z5	Zn	Yasmin (2011)
Flavobacterium sp.	Zn	He et al. (2010)
Anabaena sp.	Fe	Manjunath et al. (2016)
Azotobacter sp.	Fe, Zn	Manjunath et al. (2016)
Calothrix sp.	Zn	Manjunath et al. (2016)
Pseudomonas fluorescens C7	Fe	Vansuyt et al. (2007)

The Application of Arbuscular Mycorrhizal Fungi (AMF) to Biofortification in Crops:

Most plants, including all major food crops, form a symbiotic below-ground association with AMF that supplies the host plant with water and mineral nutrients, such as phosphorus (Senés-Guerrero et al. 2014). Plants colonized by AMF can effectively acquire nutrients from a larger soil volume, beyond the nutrition depletion zone to

where the plant roots cannot extend. Thus, AMF helps the plant to grow better and increase its productivity. In return, the host plant provides AMF with carbohydrates required to complete its life cycle (Zhang et al. 2015).

The AMF acquire essential nutrients such as P, Zn, Cu, Fe, N and K from the soil, and thus AMF contact with roots provides the plant with access to essential nutritional elements, leading to 32on-pathogenic, mobilization and uptake of the essential nutrients needed by plants for their growth (Clark and Zeto 2000; Kumar et al. 2016a). Plants colonized with AMF have significantly greater zinc concentrations in all tissue types compared to non-mycorrhizal plants, although the effect is primarily due to soil property differences (Lehmann et al. 2014). Improved total uptake of N-P-K was observed in both shoots and roots of *Plantago minuta* under variable soil water conditions, when inoculated with AMF (Shi et al. 2015). Under field conditions, inoculation of AMF in the soil of chickpea plants increased plant biomass and yield, and also increased the nutritional value of the legume, leading to Fe and Zn biofortification (Pellegrino and Bedini 2014).

In addition, AMF increases positive interactions with rhizobacteria, including beneficial rhizobacteria known to induce spore germination of AMF and root colonization directly. AMF hyphal exudates influence surrounding microbial communities and may

drive beneficial bacterial-plant interactions and consequently improve plant fitness and productivity (Scheublin et al. 2010; Cruz and Ishii 2011; Qin et al. 2016). Co-inoculation of AMF and beneficial bacteria, such as *Pseudomonas* strains, substantially increases wheat yield and mineral nutrient concentrations of P, K, Cu, Fe, Zn and Mn in wheat grains (Mader et al. 2011). The co-inoculation of selenobacteria and AMF significantly improves selenium (Se) content in wheat grain, suggesting a synergistic effect between these microbes (Durán et al. 2013). Dual inoculation of AMF and *Rhizobium* in the soybean/maize intercropping system significantly increased the efficiency of N fixation of soybean and enhanced the transfer of N from soybean to maize, leading to the yield enhancement of both crops (Meng et al. 2015).

Another important benefit of AMF is its secretion of a high molecular weight glycoprotein that helps in soil aggregation stability, promotes a better soil structure and healthy plant-soil system (Wu et al. 2014) and mitigates against phytoremediation of Al, As, Cd, Hg and Pb (He and Nara 2007).

The AMF also show resistance to several biotic (e.g. pathogen) and abiotic stressors (e.g. drought). Under drought stress, a significant increase in plant growth and photosynthesis was observed when citrus plants were colonized by AMF (Wu et al. 2013). In another study, the AMF- inoculated strawberry has shown enhanced

tolerance to drought stress (Boyer et al. 2015). AMF are capable of significantly improving plant innate system, mainly through the priming mechanism (Gallou et al. 2011).

The tomato plants colonized with AMF showed increased plant resistance to early blight, which requires JA signalling pathway (Song et al. 2015). Beneficial microbes provide plants with essential nutrients through a variety of mechanisms, and microbe interactions with plant roots are critical for nutrient uptake and crop productivity. Hence, a better understanding of microbial interactions and subsequent controlling of pathogens are essential for the use of microbes in agriculture.

The role of AMF in nutrient uptake and crop production is a key factor for biofortification of crops using essential mineral elements from the soil. With plant- microbial interactions, crops benefit through symbiosis with AMF, and this could help minimize the application of chemical fertilizers and improve nutrient availability. As a result, AMF could play a vital role in biofortification of crops with essential nutrients, thereby contributing to sustainable crops with improved nutrition that could curb global malnutrition (He and Nara 2007).

Increasing the nutritional levels of economically important food crops, without requiring the consumption of more food, is achievable through the use of AMF and other beneficial microbes.

Thus, unravelling the potential of AMF as a biofortification agent to increase the nutritional content in edible tissue plant parts is essential in the context of modern agriculture. As such, biofortification using AMF and other beneficial microbes in agriculture is important, including an increase of iron biofortification, zinc biofortification, provitamin A carotenoid biofortification and amino acid and protein biofortification of economically important crops, such as potato, sorghum, cassava, rice, wheat, maize, etc. (Mayer et al. 2008; Khush et al. 2012).

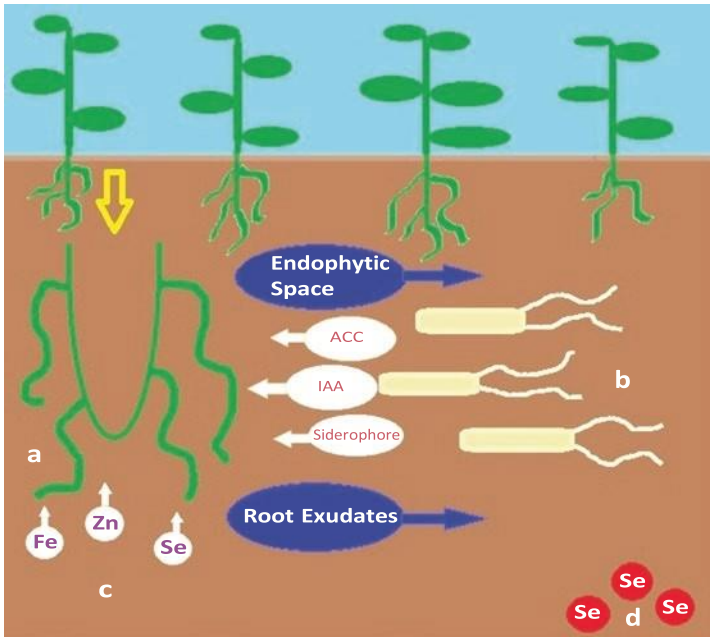


Fig. 2. Schematic representation of the reciprocal relationship between PGPR and the host plant; (**a**) represents the plant rhizospheric space, (**b**) represents beneficial PGPR. The plant provides root exudates which are consumed by bacteria as a carbon source, and endophytic PGPR have the option to potentially colonize the plant and avoid adverse environmental conditions. (**c**) Represents the increased uptake of micronutrients in the rhizosphere where PGPR activity leads to micronutrient biofortification of the plant. (**d**) Represents micronutrients in the soil which would be either unavailable or diminished in available supply to the plant without PGPR. Based on numerous PGP activities, PGPR can increase the translocation and concentration of key micronutrients which would otherwise be unavailable to the plant. Especially in the case of siderophore- producing PGPR, which can have a beneficial effect on Fe sequestration from the soil and subsequent transfer to the plant. In return, the plant can supply the PGPR with a carbon source in the form of root exudates and where there are endophytic PGPR present; the plant endosphere can act as a suitable environment for endophytic proliferation.

Conclusions and Future Perspectives:

The threats to global food security have been highlighted by many authors to date e.g. Tai et al. (2014) and Sundström et al. (2014). National governments through the international forum of the United Nations have agreed on 17 goals to be achieved by 2030 (Sustainable Development Goals project), amongst them the

eradication of world hunger and improved nutrition (FAO 2016). A number of these goals, particularly in relation to food security, can be addressed by crop biofortification. Many successful strategies for biofortifying crops are detailed in the peer-reviewed literature with the most famous biofortification project concerning the expression of provitamin A in Golden Rice which was based on a genetic engineering strategy. Even though the Golden Rice project was awarded the Patents for Humanity prize by the United States Patent and Trademark Office in 2015 in recognition of this lifesaving technology and the humanitarian nature of the project, the product is still not available on the market due to public opposition, in the main led by Greenpeace. This year (2016), 121 Nobel Laureates called on Greenpeace to end its opposition to this lifesaving technology (Support Precision Agriculture 2016).

Aside from genetic modification, other successful strategies including conventional breeding strategies have been utilized for crop biofortification. This year (2016), the World Food Prize was awarded to a group of scientists in recognition of their role in crop biofortification using breeding strategies to increase provitamin A in sweet potato by incorporating germplasm from the Andean region in South America to generate orange-fleshed sweet potatoes suitable for Africa (ISAAA 2016). This breeding strategy is reliant on natural diversity in the germplasm of a crop for the production of a particular vitamin or micronutrient.

In the absence of natural germplasm diversity, other strategies for crop biofortification can be utilized including foliar application of micronutrients and/or the exploitation of the natural microbial diversity in soils, i.e. plant growth-promoting microbes (PGPM) most especially bacteria and mycorrhizal fungi. Being environmentally safe and cost-effective are major advantages to the proliferation of PGPM throughout agriculture. Indeed this can be seen by the vast number of commercially available PGPM products released in recent years with up to 4% of the global biocide share being directly related to microbial volatile organic compounds (mVOCs) isolated and synthesized from PGPM (Glare et al. 2012; Wilson et al. 2013; Velivelli et al., 2014; Kanchiswamy et al. 2015), and this trend is set to continue. A historic problem in the biofortification of crops using foliar and soil application of micronutrients particularly in relation to Fe has been the low uptake of the micronutrient post-application. This has been highlighted recently by Kromann et al. (2016) with regard to Fe and Zn biofortification of Andean potatoes. It was found that both foliar and soil application of Zn fertilizers led to a ~three- and twofold increase, respectively, in tuber Zn concentration. However, there was no increase in Fe tuber concentration post-application. In this instance, it can be suggested that application of Fe fertilizer in conjunction with a PGPM capable of increasing Fe translocation and concentration in the tuber could be tested. The study also highlights the need to

understand soil parameters within diverse environments, as these can have a significant effect on crop biofortification (Kromann et al. 2016).

Regulatory mechanisms in the plant-microbe micro-/macronutrient cycling pathways and their reciprocal interactions need to be further elucidated to determine the best course of action for formulation of appropriate treatments to specific soil/climate/temperature/crop scenarios. Indeed, this can be further seen in the observation that nitrogen status of the plant can have a positive correlation on the content and translocation of Fe and Zn in plants (Kutman et al. 2010, 2011), and many plant growth-promoting microbes are capable of fixing atmospheric nitrogen (Prasanna et al. 2012). Genome mining of selected PGPR or wild relative/stress-tolerant cultivars of major crops may reveal genes involved in nutrient cycling pathways that, through GM technology, could be incorporated into agriculturally important crops to optimize delivery of micronutrients to deficient populations in the developing world.

Using genomics, identifying changes in gene expression during microbial interactions is critical to understanding the role of microbes in biofortification. In particular, more attention needs to be focused on the following research questions: how microbes influence the nutritional content of economically important crops; identifying appropriate microbial strains; exploiting synergistic

microbial activity, e.g. between AMF and other beneficial bacteria; and assessing constant field efficacy under different environmental regimes, to ensuring adequate uptake by plants and improving crop quality. Supplying essential nutrients using beneficial microbes naturally found in soils across the planet, such as AMF and PGPR, will help to alleviate the problem of *hidden hunger* and provide a promising sustainable agricultural strategy for improving current crop micronutrient content and in developing future biofortified crops.

References:

Acuña JJ, Jorquera MA, Barra PJ, Crowley DE, de la Luz MM (2013) Selenobacteria selected from the rhizosphere as a potential tool for Se biofortification of wheat crops. Biol Fertil Soils 49:175–185.

Adak A, Prasanna R, Babu S, Bidyarani N, Verma S, Pal M, Shivay YS, Nain L (2016) Micronutrient enrichment mediated by plant-microbe interactions and rice cultivation practices. J Plant Nutr 39:1216–1232.

Adesemoye AO, Kloepper JW (2009) Plant-microbes interactions in enhanced fertilizer-use efficiency. Appl Microbiol Biotechnol 85:1–12

Ahmad M, Nadeem SM, Naveed M, Zahir ZA (2016) Potassium-solubilizing bacteria and their application in agriculture. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 293–313.

Amanullah SA, Iqbal A, Fahad S (2016) Foliar phosphorus and zinc application improve growth and productivity of maize (*Zea mays* L.) under moisture stress conditions in semi-arid climates. J Microb Biochem Technol 8:433–439.

Bahadur I, Meena VS, Kumar S (2014) Importance and application of potassic biofertilizer in Indian agriculture. Int Res J Biol Sci 3:80–85

Bahadur I, Maurya BR, Kumar A, Meena VS, Raghuwanshi R (2016a) Towards the soil sustainability and potassium-solubilizing microorganisms. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 225–266.

Bahadur I, Maurya BR, Meena VS, Saha M, Kumar A, Aeron A (2016b) Mineral release dynamics of tricalcium phosphate and waste muscovite by mineral-solubilizing rhizobacteria isolated from indo-gangetic plain of India. Geomicrobiol J 34:454-66.

Baliga MS, Diwadkar-Navsariwala V, Koh T, Fayad R, Fantuzzi G, Diamond AM (2008) Selenoprotein deficiency enhances radiation-induced micronuclei formation. Mol Nutr Food Res 52:1300–1304.

Beard JL (2008) Why iron deficiency is important in infant development. J Nutr 138:2534–2536

Bell PF, Parker DR, Page AL (1992) Contrasting selenite sulfate interactions in se accumulating and nonaccumulating plant species. Soil Sci Soc Am 56:1818–1824.

Berutti A, Lumini E, Balestrini R, Bianciotto V (2016) Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. Front Microbiol 6:1559.

Bhutta ZA (2008) Micronutrient needs of malnourished children. Curr Opin Clin Nutr Metab Care 11:309–314

Black RE, Lindsay HA, Bhutta ZA, Caulfield LE, De Onnis M, Ezzati M, Mathers C, Rivera J (2008) Maternal and child undernutrition: global and regional exposures and health consequences. Lancet 371:243–260

Boukhalfa H, Lack JG, Reilly SD, Hersman L, Neu MP (2003) Siderophore production and facilitated uptake of iron and plutonium in *P. putida*. No. LA-UR-03-0913. Los Alamos National Laboratory

Boyer LR, Brain P, Xu XM, Jeffries P (2015) Inoculation of drought-stressed strawberry with a mixed inoculum of two arbuscular mycorrhizal fungi: effects on population dynamics of fungal species in roots and consequential plant tolerance to water deficiency. Mycorrhiza 25:215– 227.

Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao F-J, Breward N, Harriman M, Tucker M (2006) Biofortification of UK food crops with selenium. Proc Nutr Soc 65:169–181

Broadley MR, White PJ, Zelko I, Lux A (2007) Zinc in plants. New Phytol 173:677–702

Bulgarelli D, Schlaeppi K, Spaepen S, van Themaat EVL, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807–838.

Cakmak I (2008) Enrichment of cereal grains with zinc: agronomic or genetic bio-fortification? Plant Soil 302:1–17

Carlson BA, Yoo MH, Tobe R, Mueller C, Naranjo-Suarez S, Hoffmann VJ, Gladyshev VN, Hatfield DL (2012) Thioredoxin reductase 1 protects against chemically induced hepatocarcinogenesis *via* control of cellular redox homeostasis. Ox J Carcin 33:1806–1803.

Chen WW, Yang JL, Qin C, Jin CW, Mo JH, Ye T, Zheng SJ (2010) Nitric oxide acts down-stream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in *Arabidopsis*. Plant Physiol 154:810–819

Clark RB, Zeto SK (2000) Mineral acquisition by arbuscular mycorrhizal plants. J Plant Nutr 23:867–902.

Crowley DA (2006) Microbial siderophores in the plant rhizosphere. In: Barton LL, Abadia J (eds) Iron nutrition in plants and rhizospheric microorganisms. Springer, Netherlands, pp 169–189

Cruz AF, Ishii T (2011) Arbuscular mycorrhizal fungal spores host bacteria that affect nutrient biodynamics and biocontrol of soil-borne plant pathogens. Biol Open 1:52–57.

Das I, Pradhan M (2016) Potassium-solubilizing microorganisms and their role in enhancing soil fertility and health. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 281–291.

De Souza M, Chu D, Zhao M, Zayed AM, Ruzin SE, Schichnes D, Terry N (1999) Rhizosphere bacteria enhance selenium accumulation and volatilization by Indian mustard. Plant Physiol 119:565–573

Dominguez-Nunez JA, Benito B, Berrocal-Lobo M, Albanesi A (2016) Mycorrhizal fungi: role in the 43on-pathogenic of potassium. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 77–98.

Dotaniya ML, Meena VD, Basak BB, Meena RS (2016) Potassium uptake by crops as well as microorganisms. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 267–280.

Durán P, Acuña JJ, Jorquera MA, Azcón R, Borie F, Cornejo P, Mora ML (2013) Enhanced selenium content in wheat grain by co-inoculation of selenobacteria and arbuscular mycorrhizal fungi: a preliminary study as a potential Se biofortification strategy. J Cereal Sci 57:275– 280.

Durán P, Acuña JJ, Jorquera MA, Azcón R, Paredes C, Rengel Z, de la Luz MM (2014) Endophytic bacteria from selenium-supplemented wheat plants could be useful for plant-growth promotion, biofortification and *Gaeumannomyces graminis* biocontrol in wheat production. Biol Fertil Soils 50:983–990

Ellis DR, Salt DE (2003) Plants, selenium and human health. Curr Opin Plant Biol 6:273–279.

FAO (2016) The state of food and agriculture. https://www.fao.org/publications/sofa/2016/en/

Gallou A, Lucero Mosquera HP, Cranenbrouck S, Suárez JP, Declerck S (2011) Mycorrhiza induced resistance in potato plantlets challenged by *Phytophthora infestans*. Physiol Mol Plant Pathol 76:20–26.

Gamalero E, Glick B (2011) Mechanisms used by plant growth-promoting bacteria. In: Maheshwari DK (ed) Bacteria in agrobiology: plant nutrient management. Springer, Berlin, pp 17–46

Ganther HE, Levander OA, Saumann CA (1966) Dietary control of selenium volatilisation in the rat. J Nutr 88:55–60

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L, Stewart A (2012) Have biopesticides come of age? Trends Biotechnol 30:250–258.

Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica 2012:1–15

Golden Rice (2016). www.goldenrice.org

Gopalakrishnan S, Vadlamundi S, Samineni S, Sameer Kumar CV (2016) Plant growth-promotion of chickpea and pigeon pea through inoculation of biocontrol potential bacteria, isolated from organic soils. Springer Plus 5:1882.

Hafeez FY, Hameed S, Zaidi AH, Malik KA (2002) Biofertiliser for sustainable agriculture. In: Azam F, Iqbal MM, Inayatullah C, Malik KA (eds) Technologies for sustainable agriculture. NIAB, Faisalabad, pp 67–74

Harvest Plus (2016). http://www.harvestplus.org/biofortification-nutrition-revolution-now

Hatfield DL, Tsuji PA, Carlson BA, Gladyshev VN (2014) Selenium and selenocysteine: roles in cancer, health and development. Trends Biochem Sci 39:112–120.

Hawkesford MJ, Zhao F-J (2007) Strategies for increasing the selenium content of wheat. J Cereal Sci 46:282–292

He X, Nara K (2007) Element biofortification: can mycorrhizas potentially offer a more effective and sustainable pathway to curb human malnutrition? Trends Plant Sci 12:331–333.

He CQ, Tan G, Liang X, Du W, Chen YL, Zhi GY, Zhu Y (2010) Effect of Zn-tolerant bacterial strain on growth and Zn accumulation in *Orychophragmus violaceus*. Appl Soil Ecol 44:1–5

International Rice Research Institute (2016). http://irri.org/golden-rice/faqs

ISAAA (2016). http://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=14884

Jaiswal DK, Verma JP, Prakash S, Meena VS, Meena RS (2016) Potassium as an important plant nutrient in sustainable agriculture: a state of the art. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 21–29.

Jat LK, Singh YV, Meena SK, Meena SK, Parihar M, Jatav HS, Meena RK, Meena VS (2015) Does integrated nutrient management enhance agricultural productivity? J Pure Appl Microbiol 9:1211–1221

Jha Y, Subramanian RB (2016) Regulation of plant physiology and antioxidant enzymes for alleviating salinity stress by potassium-mobilizing bacteria. In: Meena VS, Maurya BR,

Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 149–162

Jin CW, He YF, Tang CX, Wu P, Zheng SJ (2006) Mechanisms of microbially enhanced iron uptake in red clover. Plant Cell Environ 29:888–897

Jin CW, You GY, Zheng SJ (2008) The iron-deficiency induced phenolics secretion plays multiple important roles in plant iron acquisition underground. Plant Signal Behav 3:60–61

Kanchiswamy CN, Malnoy M, Maffei ME (2015) Bioprospecting bacterial and fungal volatiles for sustainable agriculture. Trends Plant Sci 20:206–211

Khush GS, Lee S, Cho J-I, Jeon J-S (2012) Biofortification of crops for reducing malnutrition. Plant Biotechnol Reports 6:195–202

King JC (2006) Zinc. In: Shills ME, Shike M (eds) Modern nutrition in health and disease, 10th edn. Lippincott Williams and Wilkins, Philadelphia, pp 271–285

King JC (2011) Zinc: an essential but elusive nutrient. Am J Clin Nutr 94(suppl):697S–684S

Kloepper JW, Schroth MN, Miller TD (1980) Effects of rhizosphere colonization by plant growth- promoting rhizobacteria on potato plant development and yield. Phytopathology 70:1078–1082

Krishnakumar S, Balakrishnan N, Muthukrisnan R, Kumar SR (2013) Myth and mystery of soil mycorrhiza: a review. Af J Ag Res 8:4706–4717

Kromann P, Valverde F, Alvardo S, Vélez R, Pisuña J, Potosí B, Taipe A, Caballero D, Cabezas A, Devaux A (2017) Can Andean potatoes be agronomically biofortified with iron and zinc fertilisers? Plant Soil 411:121-138

Kumar A, Bahadur I, Maurya BR, Raghuwanshi R, Meena VS, Singh DK, Dixit J (2015) Does a plant growth-promoting rhizobacteria enhance agricultural sustainability? J Pure Appl Microbiol 9:715–724

Kumar A, Choudhary AK, Pooniya V, Suri VK, Singh U (2016a) Soil factors associated with micronutrient acquisition in crops-biofortification perspective. In: Singh U, Praharaj SC, Singh SS, Singh PN (eds) Biofortification of food crops. Springer, India, pp 159–176

Kumar A, Meena R, Meena VS, Bisht JK, Pattanayak A (2016b) Towards the stress management and environmental sustainability. J Clean Prod 137:821–822

Kumar A, Patel JS, Bahadur I, Meena VS (2016c) The molecular mechanisms of KSMs for enhancement of crop production under organic farming. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 61–75

Kumar A, Maurya BR, Raghuwanshi R, Meena VS, Islam MT (2017) Co-inoculation with Enterobacter and Rhizobacteria on yield and nutrient uptake by wheat (*Triticum aestivum* L.) in the alluvial soil under indo-gangetic plain of India. J Plant Growth Regul 36:608-617

Kutman UB, Yildiz B, Ozturk L, Cakmak I (2010) Biofortification of durum wheat with zinc through soil and foliar application of nitrogen. Cereal Chem 87:1–9

Kutman UB, Yildiz B, Cakmak I (2011) Improved nitrogen status enhances zinc and iron concentrations both in the whole grain and the endosperm fraction of wheat. J Cereal Sci 53:118–125

Lehmann A, Veresoglou SD, Leifheit EF, Rillig MC (2014) Arbuscular mycorrhizal influence on zinc nutrition in crop plants – a meta-analysis. Soil Biol Biochem 69:123–131

Li SH, Xiao TF, Zheng BS (2012) Medical geology of arsenic, selenium and thallium in China. Sci Total Environ 421–422:31–40

Lugtenberg B, Kamilova F (2009) Plant growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556

Mäder P, Kaiser F, Adholeya A, Singh R, Uppal HS, Sharma AK, Srivastava R, Sahai V, Aragno M, Wiemken A, Johri BN, Fried PM (2011) Inoculation of root microorganisms for sustainable wheat–rice and wheat–black gram rotations in India. Soil Biol Biochem 43:609–619

Manjunath M, Kanchan A, Ranjan K, Venkatachalam S, Prasanna R, Ramakrishnan B, Hossain F, Nain L, Shivay YS, Rai AB, Singh B (2016) Beneficial cyanobacteria and eubacteria synergistically enhance bioavailability of soil nutrients and yield of Okra. Heliyon 2:e00066

Masalha J, Kosegarten H, Elmaci Ö, Mengel K (2000) The central role of microbial activity for iron acquisition in maize and sunflower. Biol Fertil Soils 30:433–439

Masood S, Bano A (2016) Mechanism of potassium 48on-pathogenic in the agricultural soils by the help of soil microorganisms. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 137– 147

Maurer B, Keller-Scheirlin W (1968) Ferribactin, a siderophore from *Pseudomonas fluorescens* Migula 61. Arch Mirobiol 60:326–339

Maurya BR, Meena VS, Meena OP (2014) Influence of inceptisol and alfisol's potassium solubilizing bacteria (KSB) isolates on release of K from waste mica. Vegetos 27:181–187

Mayer JE, Pfeiffer WH, Beyer P (2008) Biofortified crops to alleviate micronutrient malnutrition. Curr Opin Plant Biol 11:166–170

McConnell KP, Portman OW (1952) Toxicity of dimethyl selenide in the rat and mouse. Proc Soc Exp Biol Med 79:230–231

Meena OP, Maurya BR, Meena VS (2013a) Influence of K-solubilizing bacteria on release of potassium from waste mica. Agric Sust Dev 1:53–56

Meena VS, Maurya BR, Bohra JS, Verma R, Meena MD (2013b) Effect of concentrate manure and nutrient levels on enzymatic activities and microbial population under submerged rice in alluvium soil of Varanasi. Crop Res 45(1,2 & 3):6–12

Meena VS, Maurya BR, Verma R, Meena RS, Jatav GK, Meena SK, Meena SK (2013c) Soil microbial population and selected enzyme activities as influenced by concentrate manure and inorganic fertilizer in alluvium soil of Varanasi. Bioscan 8:931–935

Meena VS, Maurya BR, Bahadur I (2014a) Potassium 49on-pathogenic by bacterial strain in waste mica. Bang J Bot 43:235–237

Meena VS, Maurya BR, Verma JP (2014b) Does a rhizospheric microorganism enhance K^+ availability in agricultural soils? Microbiol Res 169:337–347

Meena RS, Meena VS, Meena SK, Verma JP (2015a) The needs of healthy soils for a healthy world. J Clean Prod 102:560–561

Meena RS, Meena VS, Meena SK, Verma JP (2015b) Towards the plant stress mitigate the agricultural productivity: a book review. J Clean Prod 102:552–553

Meena VS, Maurya BR, Verma JP, Aeron A, Kumar A, Kim K, Bajpai VK (2015c) Potassium solubilizing rhizobacteria (KSR): isolation, identification, and K-release dynamics from waste mica. Ecol Eng 81:340–347

Meena VS, Meena SK, Verma JP, Meena RS, Ghosh BN (2015d) The needs of nutrient use efficiency for sustainable agriculture. J Clean Prod 102:562–563

Meena VS, Maurya BR, Meena RS (2015e) Residual impact of wellgrow formulation and NPK on growth and yield of wheat (*Triticum aestivum* L.) Bangladesh J Bot 44:143–146

Meena VS, Verma JP, Meena SK (2015f) Towards the current scenario of nutrient use efficiency in crop species. J Clean Prod 102:556–557

Meena RK, Singh RK, Singh NP, Meena SK, Meena VS (2016a) Isolation of low temperature surviving plant growth-promoting rhizobacteria (PGPR) from pea (*Pisum sativum* L.) and documentation of their plant growth promoting traits. Biocatal Agric Biotechnol 4:806–811

Meena VS, Bahadur I, Maurya BR, Kumar A, Meena RK, Meena SK, Verma JP (2016b) Potassium- solubilizing microorganism in evergreen agriculture: an overview. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 1–20

Meena VS, Meena SK, Bisht JK, Pattanayak A (2016c) Conservation agricultural practices in sustainable food production. J Clean Prod 137:690–691

Meena RS, Bohra JS, Singh SP, Meena VS, Verma JP, Verma SK, Sihag SK (2016d) Towards the prime response of manure to enhance nutrient use efficiency and soil sustainability a current need: a book review. J Clean Prod 112:1258–1260

Meena SK, Rakshit A, Meena VS (2016e) Effect of seed bio-priming and N doses under varied soil type on nitrogen use efficiency (NUE) of wheat (*Triticum aestivum* L.) under greenhouse conditions. Biocatal Agric Biotechnol 6:68–75

Meng L, Zhang A, Wang F, Han X, Wang D, Li S (2015) Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. Front Plant Sci 6:339

Meplan C, Hesketh J (2012) The influence of selenium and selenoprotein gene variants on colorectal cancer risk. Mutagenesis 27:177–186

Nakandalage N, Nicolas M, Norton RM, Hirotsu N, Milham PJ, Seneweera S (2016) Improving rice zinc biofortification success rates through genetic and crop management approaches in a changing environment. Front Plant Sci 7:764

Nuberger A, Okebe J, Yahav D, Paul M (2016) Oral iron supplements for children in malaria- endemic areas. Cochrane Database Syst Rev 1–129

Parewa HP, Yadav J, Rakshit A, Meena VS, Karthikeyan N (2014) Plant growth promoting rhizobacteria enhance growth and nutrient uptake of crops. Agric Sustain Dev 2:101–116

Pellegrino E, Bedini S (2014) Enhancing ecosystem services in sustainable agriculture: Biofertilization and biofortification of chickpea (*Cicer arietinum* L.) by arbuscular mycorrhizal fungi. Soil Biol Biochem 68:429–439

Prakash S, Verma JP (2016) Global perspective of potash for fertilizer production. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 327–331

Prasad AS (2008) Zinc in human health: effect of zinc on immune cells. Mol Med 14:357

Prasanna R, Joshi M, Rana A, Shivay YS, Nain L (2012) Influence of co-inoculation of bacteria- cyanobacteria on crop yield and C-N sequestration in soil under rice crop. World J Microbiol Biotechnol 28:1223–1235

Priyadharsini P, Muthukumar T (2016) Interactions between arbuscular mycorrhizal fungi and potassium-solubilizing microorganisms on agricultural productivity. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 111–125

Qin H, Brookes PC, Xu J (2016) Arbuscular mycorrhizal fungal hyphae alter soil bacterial community and enhance polychlorinated biphenyls dissipation. Front Microbiol 7:939

Raghavendra MP, Nayaka NC, Nuthan BR (2016) Role of rhizosphere microflora in potassium 51on-pathogenic. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 43–59

Rana A, Joshi M, Prasanna R, Shivay YS, Nain L (2012) Biofortification of wheat through inoculation of plant growth promoting rhizobacteria and cyanobacteria. Eur J Soil Biol 50:118–126

Rana A, Kabi SR, Verma S, Adak A, Madan P, Shivay YS, Prasanna R and Nain L (2015) Prospecting plant growth promoting bacteria and cyanobacteria as options for enrichment of macro- and micronutrients in grains in rice-wheat cropping sequence. Cogent Food Agric 1: 1037379

Rawat J, Sanwal P, Saxena J (2016) Potassium and its role in sustainable agriculture. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 235–253

Saha M, Maurya BR, Meena VS, Bahadur I, Kumar A (2016a) Identification and characterization of potassium solubilizing bacteria (KSB) from Indo-Gangetic Plains of India. Biocatal Agric Biotechnol 7:202–209

Saha M, Sarkar S, Sarkar B, Sharma BK, Bhattacharjee S, Tribedi P (2016b) Microbial siderophores and their potential applications: a review. Environ Sci Pollut Res 23:3984–3999

Saha M, Maurya BR, Bahadur I, Kumar A, Meena VS (2016c) Can potassium-solubilising bacteria mitigate the potassium problems in India? In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 127–136

Sathya A, Vijayabharathi R, Srinivas V, Gopalakrishnan S (2016) Plant growth-promoting actinobacteria on chickpea seed mineral density: an upcoming complementary tool for sustainable biofortification strategy. 3 Biotech 6:138

Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A, Dhingra U, Kabole I, Deb S, Othman MK, Kabole FM (2006) Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367:133–143

Schalk IJ, Guillon L (2013) Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: implications for metal homeostasis. Environ Microbiol 15:1661–1673

Scheublin TR, Sanders IR, Keel C, van der Meer JR (2010) Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi. ISME J 4:752–763

Senés-Guerrero C, Torres-Cortés G, Pfeiffer S, Rojas M, Schüßler A (2014) Potato-associated arbuscular mycorrhizal fungal communities in the Peruvian Andes. Mycorrhiza 24:405– 417

Shakeel M, Afroz R, Hssan MN, Hafeez FY (2015) Root associated Bacillus sp. improves growth, yield and zinc translocation for basmati rice (*Oryza sativa*) varieties. Front Microbiol 6:1286

Sharma A, Shankhdhar D, Shankhdhar SC (2016) Potassium-solubilizing microorganisms: mechanism and their role in potassium 52on-pathogenic and uptake. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 203–219

Shi Z, Mickan B, Feng G, Chen Y (2015) Arbuscular mycorrhizal fungi improved plant growth and nutrient acquisition of desert ephemeral *Plantago minuta* under variable soil water conditions. J Arid Land 7:414–420

Shrivastava M, Srivastava PC, D'Souza SF (2016) KSM soil diversity and mineral 53on-pathogenic, in relation to crop production and molecular mechanism. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 221–234

Siddiqui ZA (2006) PGPR: prospective biocontrol agents of plant pathogens. In: Siddiqui ZA (ed) PGPR: biocontrol and biocontrol. Springer, Dordrecht, pp 112–142

Sindhu SS, Parmar P, Phour M, Sehrawat A (2016) Potassium-solubilizing microorganisms (KSMs) and its effect on plant growth improvement. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 171–185

Singh NP, Singh RK, Meena VS, Meena RK (2015) Can we use maize (*Zea mays*) rhizobacteria as plant growth promoter? Vegetos 28:86–99

Singh M, Dotaniya ML, Mishra A, Dotaniya CK, Regar KL, Lata M (2016) Role of biofertilizers in conservation agriculture. In: Bisht JK, Meena VS, Mishra PK, Pattanayak A (eds) Conservation agriculture: an approach to combat climate change in Indian Himalaya. Springer, Singapore, pp 113–134

Smith MJ, Neilands JB (1984) Rhizobactin, a siderophore from *Rhizobium meliloti*. J Plant Nutr 7:449–458

Song Y, Chen D, Lu K, Sun Z, Zeng R (2015) Enhanced tomato disease resistance primed by arbuscular mycorrhizal fungus. Front Plant Sci 6:786

Sors TG, Ellis DR, Salt DE (2005) Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynth Res 86:373–389

Srivastava RP (2016) Antinutrients restraining biofortification. In: Singh U, Praharaj CS, Singh SS, Singh NP (eds) Biofortification of food crops. Springer, India, pp 333–348

Sunde RA (2012) Selenium. In: Ross AC, Caballero B, Cousins RJ, Tucker KL, Ziegler TR (eds) Modern nutrition in health and disease, 11th edn. Lippincott Williams and Wilkins, Philadelphia, pp 225–237

Sundström JF, Albihn A, Boqvist S, Ljungvall K, Marstrop H, Martiin C, Nyberg K, Vågsholm I, Yuen J, Magnusson U (2014) Future threats to agricultural food production posed by environmental degradation, climate change, and animal and plant diseases – a risk analysis in three economic and climate settings. Food Sec 6:201

Support Precision Agriculture (2016). http://supportprecisionagriculture.org/

Sura-de Jong M, Reynolds RJB, Richterova K, Musilova L, Staicu LC, Chocholata I, Cappa JJ, Taghavi S, van der Lelie D, Frantik T, Dolinova I, Strejcek M, Cochran AT, Lovecka P, Pilon- Smits EAH (2015) Selenium hyperaccumulators 54on-pat a diverse endophytic bacterial community characterized by high selenium resistance and plant growth promoting properties. Front Plant Sci 6:113

Tai APK, Martin MV, Heald CL (2014) Threat to future global food security from climate and ozone air pollution. Nat Clim Chang 4:817–821

Tariq M, Hameed S, Malik KA, Hafeez FY (2007) Plant root associated bacteria for zinc mobilisation in rice. Pak J Bot 39:245–253

Teotia P, Kumar V, Kumar M, Shrivastava N, Varma A (2016) Rhizosphere microbes: potassium solubilisation and crop productivity-present and future aspects. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 315–325

Terry N, Zayed AM, de Souza MP, Tarun AS (2000) Selenium in higher plants. Annu Rev Plant Physiol Plant Mol Biol 51:401–432

Trijatmiko KR, Dueñas C, Tsakirpaloglou N, Torrizo L, Arines FM, Adeva C, Balindong J, Oliva N, Sapasap MV, Borrero J, Rey J, Francisco P, Nelson A, Nakanishi H, Lombi E, Tako E, Glahn RP, Stangoulis J, Chadha-Mohanty P, Johnson AAT, Tohme J,

Barry G, Slamet-Lohdin IH (2016) Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. Sci Rep 6:19792

UN (2015) Sustainable development knowledge platform. https://sustainabledevelopment.un.org/ about

Urbano G, Lopez-Jurado M, Aranda P, Vidal-Valverde C, Tenorio E, Porres J (2000) The role of phytic acid in legumes: antinutrient or beneficial function. J Physiol Biochem 56:283–229

Vansuyt G, Robin A, Briat JF, Curie C, Lemanceau P (2007) Iron acquisition from Fe-pyoverdine by *Arabidopsis thaliana*. Mol Plant Microbe Interact J 20:441–447

Velazquez E, Silva LR, Ramírez-Bahena MH, Peix A (2016) Diversity of potassium- solubilizing microorganisms and their interactions with plants. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 99–110

Velivelli SLS, Sessitch A, Doyle Prestwich B (2014) The role of microbial inoculants in integrated crop management systems. Potato Res 57:291–309

Verma R, Maurya BR, Meena VS (2014) Integrated effect of bio-organics with chemical fertilizer on growth, yield and quality of cabbage (*Brassica oleracea* var *capitata*). Indian J Agric Sci 84:914–919

Verma JP, Jaiswa DK, Meena VS, Meena RS (2015a) Current need of organic farming for enhancing sustainable agriculture. J Clean Prod 102:545–547

Verma JP, Jaiswal DK, Meena VS, Kumar A, Meena RS (2015b) Issues and challenges about sustainable agriculture production for management of natural resources to sustain soil fertility and health. J Clean Prod 107:793–794

Wang U, Yang X, Zhang X, Dong L, Zhang J, Wei Y, Feng Y, Lu L (2014) Improved plant growth and Zn accumulation in grains of rice (*Oryza sativa* L.) by inoculation of endophytic microbes isolated from a Zn hyperaccumulator, *Sedum alfredii* H. J Agric Food Chem 62:1783–1791

Weinberg ED (1999) The role of iron in fungal and protozoan infectious diseases. J Eukaryot Microbiol 46:231–238

Welch RM (2001) Impact of mineral nutrients in plants on human nutrition on a worldwide scale. In: Horst WJ, Schenk MK, Bürkert A (eds) Plant nutrition-food security and sustainability of agro-ecosystems. Kluwer, Dordrecht, pp 284–285

White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets: iron zinc copper calcium magnesium selenium and iodine. New Phytol 182:49–84

White PJ, Bowen HC, Parmaguru P, Fritz M, Spracklen WP, Spiby RE, Meacham MC, Mead A, Harriman M, Trueman LJ, Smith BM, Thomas B, Broadley MR (2004) Interaction between selenium and sulfur nutrition in *Arabidopsis thaliana*. J Exp Bot 55:1927–1937

White PJ, Broadley MR, Bowen HC, Johnson SE (2007) Selenium and its relationship with sulfur. In: Hawkesford MJ, de Kok LJ (eds) Sulfur in plants – an ecological perspective. Springer, London, pp 225–252

WHO (2006) Guidelines of food fortification with micronutrients. http://www.who.int/nutrition/ publications/guide_food_fortification_micronutrients.pdf

WHO (2016a) Biofortification of staple crops. http://www.who.int/elena/titles/biofortification/en/

WHO (2016b) Micronutrient deficiencies: iron deficiency anaemia. http://www.who.int/nutrition/ topics/ida/en/

Wilber CG (1980) Toxicology of selenium: a review. Clin Toxicol 17:171–230

Wilson K, Benton TG, Graham RI, Grzywacz D (2013) Pest control: biopesticides' potential. Science 342(6160):799

Wu Q-S, Srivastava AK, Zou Y-N (2013) AMF-induced tolerance to drought stress in citrus: a review. Scientia Hort 164:77–87.

Wu Q-S, Cao M-Q, Zou Y-N, He X-h (2014) Direct and indirect effects of glomalin, mycorrhizal hyphae, and roots on aggregate stability in rhizosphere of trifoliate orange. Sci Rep 4:5823

Wu T, Zhang HT, Wang Y, Jia WS, Xu XF, Zhang XZ, Han ZH (2012) Induction of root Fe (III) reductase activity and proton extrusion by iron deficiency is mediated by auxin-based systemic signalling in *Malus xiaojinensis*. J Exp Bot 63:859–870

Wu Z, Bañuelos GS, Lin ZQ, Liu Y, Yin X, Li M (2015) Biofortification and phytoremediation of selenium in China. Front Plant Sci 6:136

Yadav BK, Sidhu AS (2016) Dynamics of potassium and their bioavailability for plant nutrition. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 187–201

Yasin M, El-Medhawi AF, Jahn CE, Anwar A, Turner MFS, Faisal M, Pilon-Smits EAH (2015a) Seleniferous soils as a source for production of selenium-enriched foods and potential of bacteria to enhance plant selenium uptake. Plant Soil 386:385–394

Yasin M, El-Medhawi AF, Pilon-Smits EAH, Faisal M (2015b) Selenium-fortified wheat: potential of microbes for biofortification of selenium and other essential nutrients. Int J Phytoremediation 17:777–786

Yasin M, Munir I, Faisal M (2016) Can *Bacillus* spp. Enhance K^+ uptake in crop species. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 163–170

Yasmin S (2011) Characterization of growth promoting and bioantagonistic bacteria associated with rhizosphere of cotton and rice. NIBGE, Faisalabad

Zahedi H (2016) Growth-promoting effect of potassium-solubilizing microorganisms on some crop species. In: Meena VS, Maurya BR, Verma JP, Meena RS (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer, India, pp 31–42

Zhang H, Sun Y, Xie X, Kim MS, Dowd SE, Paré PW (2009) A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. Plant J 58:568–577

Zhang Y, Song Q, Yan J, Tang J, Zhao R, Zhang Y, He Z, Zou C, Ortiz-Moasterio I (2010) Mineral element concentrations in grains of Chinese wheat cultivars. Euphytica 174:303–313

Zhang Z-Z, Lou Y-G, Deng D-J, Rahman MM, Wu Q-S (2015) Effects of common mycorrhizal network on plant carbohydrates and soil properties in trifoliate orange–white clover association. PloS One 10:e0142371

Chapter 2

The Role of Rhizobacterial Volatile Organic Compounds in a Second Green Revolution—The Story so Far

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Abstract:

The role of microbial-emitted volatiles (mVOCs) also termed 'infochemicals' in agriculture is an emerging area of research with many perceived attributes including but not limited to the alleviation of abiotic and biotic stress factors. Several reports in the literature to date have demonstrated the potential of these mVOCs in plant growth-promotion and disease-suppression, albeit mainly under artificial conditions. The mVOCs are low

molecular mass compounds with a high vapour pressure and low boiling point and through diffusion can affect a response over a long distance both above and below ground. They belong to many different classes of chemicals that include terpenes, alcohols, alkenes and ketones amongst others. This review examines recent literature in this area and cites examples of mVOCs, or more particularly; bacterial-derived volatile compounds hereby referred to as 'BVCs', that have plant growth promoting and biocontrol effects. The multifaceted role of BVCs can be viewed as an integral part of a second green revolution in agriculture where alternative environmentally-friendly solutions are being sought for crop protection and bio-stimulation. Their ability to modulate plant photosynthetic and ISR pathways may provide the agricultural sector with more sustainable solutions for increased crop protection and production in the face of increasing climate and population changes.

Introduction:

The plant rhizosphere is a highly competitive environment with plants releasing as much as 40% of their photosynthetic carbon through their roots with these secreted nutrients enabling the creation of a 'hot-spot' of microbial activity (including

rhizobacterial activity) (Kai et al. 2016). Rhizobacteria enhance plant growth through a number of different mechanisms, some known, others as yet unknown (Velivelli et al. 2015). Over the past four decades these microbes have been commonly referred to as 'Plant Growth-Promoting Rhizobacteria' (PGPR) (Kloepper and Schroth 1978; Ryu et al. 2005a). Different mechanisms are involved in the enhancement of plant growth by rhizobacteria. Some of these mechanisms are termed direct, others indirect. Examples of direct mechanisms can include the biosynthesis of chemicals analogous to plant hormones involved in the plant growth process such as indole-3-acetic acid (Shao et al. 2015). The optimisation of plant nutrient-uptake is also facilitated through phosphorus solubilisation (Oteino et al. 2015), nitrogen fixation (Singh 2014) and/or by modulating the levels of ethylene in the plant through the activity of enzymes such as aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick 2014). Indirect mechanisms include the synthesis of non-volatile antibiotics such as pyoluterin, surfactin and fengycin (Dimkić et al. 2017); competition for nutrients mediated by siderophore production for enhanced iron-uptake from soil (Ahmed and Holmström 2014); the secretion of lytic enzymes (e.g. chitinase, b-1,3-glucanase) and the modulation of plant immunity via activation of the induced systemic resistance (ISR) pathway (Compant et al. 2005; Lugtenberg and Kamilova 2009; Velivelli et al. 2015; Tahir et al. 2017a) (Fig.1). In recent years, there has

been increased interest in the effects of rhizobacterial volatiles on plants (Weisskopf et al. 2016). The metabolic activity of the soil microbiota involves the synthesis of a broad variety of infochemicals, of which volatile organic compounds (VOCs) comprise a large proportion (Kanchiswamy et al. 2015; Velivelli et al. 2015). VOCs are characterised as having a relatively low molecular weight (<300 Da), a low boiling point and high vapour pressure (Vespermann et al. 2007; Velivelli et al. 2014). Microbial-emitted volatiles (mVOCs) belong to a number of different chemical classes including, but not limited to; alcohols, ketones, alkenes and terpenes (Schulz-Bohm 2017) and to date bacteria have been found to produce over 1000 VOCs (Sharifi and Ryu 2018a). The profile of VOCs emitted depends to a large extent on the external environment, be that soil properties or media components (Fincheira and Quiroz 2018).

Infochemicals are of great importance, because volatiles can facilitate both the intra and inter-kingdom interaction between many organisms including plants and microbes (Farag et al. 2017). Due to their capacity to disperse in the atmosphere and to circulate through permeable soil structures, volatiles can exert their effects on plants above and below ground (Sharifi and Ryu 2018a). There are two types of VOCs—organic (e.g. 2,3-butanediol) and inorganic (e.g. HCN, CO_2). The complex blends of VOCs that rhizobacteria are capable of generating have been

the focus of numerous studies over the past decade (Yuan et al. 2017; Song and Ryu 2018; Tahir et al. 2017b; Blom et al. 2011a) These VOC blends can have beneficial or detrimental effects on the growth of plants, fungi and other associated organisms within the environment of the respective VOC-emitter (Effmert et al. 2012) (Table 1). Exposure to BVCs can enhance plant growth under certain conditions, but can induce phytotoxic effects in others (Rath et al. 2018). Different blends of volatiles have been implicated in seed germination, flowering time and number, and in fruit and seed production (Sharifi and Ryu 2018a). A variety of chemical signalling molecules are produced by both rhizobacteria and plants when grown together, demonstrating that active communication exists between these kingdoms during plant development (Leach et al. 2017; Farag et al. 2017). For example, microbe-derived compounds are detected by plants, which can then adapt their defence and growth responses to specific types of microorganism. Furthermore, for the exploitation of BVCs in agronomical contexts, we need to determine both their activity and validity-for-use in the field (Rosier et al. 2018). Therefore, it is essential to have a comprehensive understanding of the biocontrol mechanisms of these agents so as to facilitate efficient and effective agronomic application.

Table 1. List of some of volatile-mediated effects of bacteria on plants, fungi and nematodes

Bacteria	Volatile metabolites	Functions	References
B. subtilis GB03,	2,3-butanediol	Growth promotion and ISR	Ryu et al. (2003, 2004); Song et al. (2019)
B. amyloliquefaciens IN937a *P. chlororaphis* O6	2,3-butanediol	Protection against drought stress	Cho et al. (2008)
B. subtilis FB17	Acetoin	Induced systemic resistance (ISR)	Rudrappa et al. (2010)
B. megaterium XTBG34	2-pentylfuran	Growth promotion	Zou et al. (2010)
A. agilis UMCV2	Dimethylhexadecylamine	Growth Promotion	Velázquez-Becerra et al. (2011)
P. polymyxa E681	Tridecane	Induced systemic resistance (ISR)	Lee et al. (2012)
B. ambifaria	Dimethyl disulphide, Acetophenone, 3-hexanone and 2,5-dimethylpyrazine	Growth promotion	Groenhagen et al. (2013)
P. vulgaris JBLS202	Indole	Growth promotion	Yu and Lee (2013)
S. plymuthica HRO-C48	Dimethyl disulphide	Fungal growth inhibition	Muller et al. (2009)
Bacillus sp.	Acetoin	Fungal pathogen inhibition	Arrebola et al. (2010)
Flavobacterium sp. GSE09 *Lysobacter* enzymogenes ISE13	2,4-di-tert-butylphenol	Fungal growth inhibition	Sang et al. (2011)
P. polymyxa BMP-11	1-octen-3-ol,benzothiazole and citronellol	Fungal growth inhibition	Zhao et al. (2011)
X. campestris pv. *Vesicatoria* 85-10	Decan-2-one	Fungal growth inhibition	Weise et al. (2012)
B. ambifaria	Dimethyl disulphide, 2-nonanone, 1-phenyl-1,2-propanedione, 2-undecanone, dimethyl trisulfide, 4-octanone, S-methylmethanethiosulphonate and acetophenone	Fungal growth inhibition	Groenhagen et al. (2013)

Rhizobacterial Volatiles and Plant Growth:

The direct physical interaction between rhizobacteria and their respective plant host underpins most PGPR-plant interactions. However, an emerging field over the past number of years has examined the long-distance relationships which plants and rhizobacteria can achieve via the medium of VOCs (Velivelli et al. 2014). The first observation of this phenomenon was by Choong-Min Ryu and co-workers and this has led to the opening of many new avenues in the field of plant–microbe interactions. Where physical contact with the plant is not possible, certain rhizobacteria rely on the production of BVCs, a classic example being the volatile alcohol, 2,3-butanediol or more specifically its stereoisomer '2R, 3R- butanediol' to stimulate plant development or activate ISR (Ryu et al. 2003, 2004; Lee et al. 2012; Fincheira and Quiroz 2018) (Fig. 1). The identification of various plant growth promoting infochemicals and the determination of their structures and their associated functions have been ground-breaking moments in the study of plant-microbe interactions and pinning down the roles of VOCs in the intricate signalling systems between plants and rhizobacteria has been the key area of interest among a number of research groups (Bailly and Weisskopf 2017; Sharifi and Ryu 2018a).

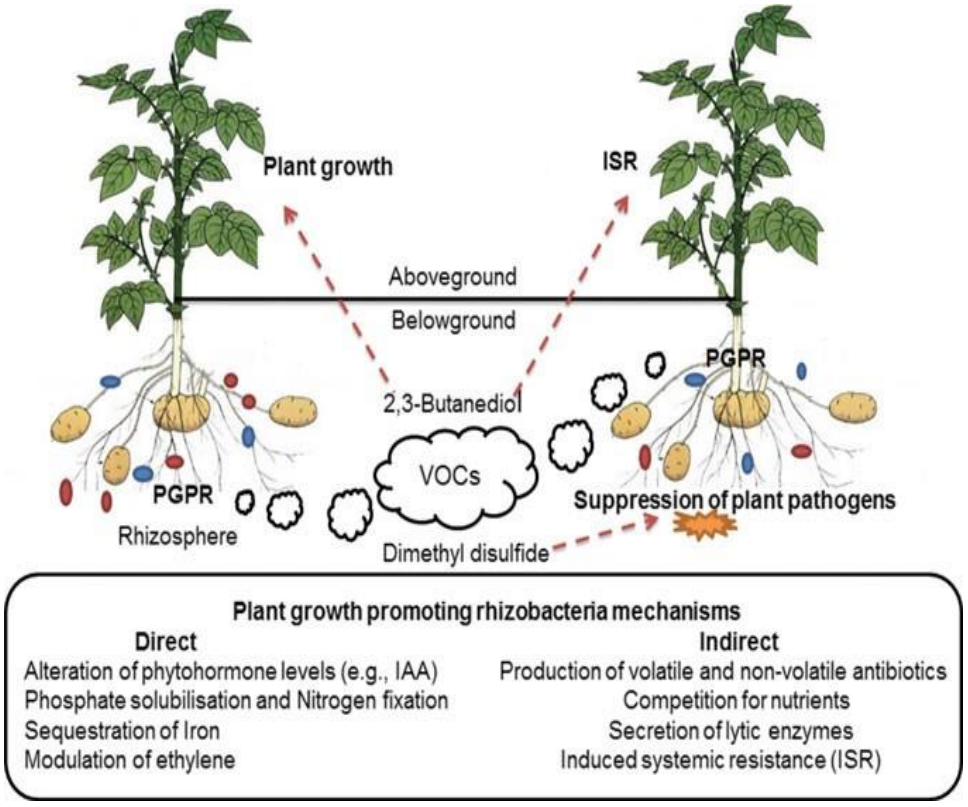


Fig. 1. Representation of interactions of bacterial volatile organic compounds (BVCs) on plants (reproduced with permission from Velivelli et al. 2014)

Nevertheless, the contribution of rhizobacterial VOCs to plant development and the importance of these compounds in agricultural systems are still topics of significant debate and speculation. In addition, only a handful of VOCs that are secreted by rhizobacteria have been identified to date. Therefore, a comprehensive understanding of the biological and ecological functions of BVCs—let alone a full understanding of their potential uses—has yet to be achieved.

However, according to Fincheira and Quiroz (2018), mVOCs can influence plant growth in 4 ways: the modulation of nutrients,

alteration of hormone levels, influencing plant metabolism and changing sugar concentrations.

Most importantly, future studies will need to address the types of responses and signalling cascades that are induced in plants by BVCs. Research focusing on the effects of plant exposure to different BVCs has uncovered a wide range of effects, including significant plant growth, the induction of ISR and even plant phytotoxicity. In particular, 2,3-butanediol (Ryu et al. 2003, 2004) dimethylhexadecylamine (Velázquez-Becerra et al. 2011), 2-pentylfuran (Zou et al. 2010), indole (Blom et al. 2011a; Yu and Lee 2013; Fincheira and Quiroz 2018) and dimethyl disulphide (DMDS) (Groenhagen et al. 2013), are amongst some of the infochemicals that have been shown to increase plant growth, whereas negative effects are at least in part due to the presence of high levels of hydrogen cyanide (HCN) (Blom et al. 2011b), DMDS and ammonia (Kai et al. 2010; Weise et al. 2013). To determine the extent to which BVCs stimulate plant growth, Choong-Min Ryu and co-workers used two-compartment Petri dishes, hereby referred to as 'I-plates', to physically separate *Arabidopsis thaliana* from rhizobacteria under laboratory conditions. In this way, the dispersal of non-volatile metabolites through the medium was prevented, allowing for only the exchange of volatile organic compounds.

The researchers observed that plant growth was most strongly stimulated by the bacterial strains *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a. When the volatiles produced by these two strains were examined, it was found that these bacteria produced the compounds 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, which were not detected in bacterial strains that were unable to induce volatile-mediated plant growth. The exogenous application of these two compounds in pure solutions induced similar effects in a dose-dependent manner, and *Bacillus* spp. Mutants defective in 2,3-butanediol and acetoin synthesis showed no plant growth-promotion, which confirmed the role of these compounds in mediating plant growth. A set of hormonal mutant *A. thaliana* lines impaired in specific regulatory pathways was then tested to identify the signalling networks necessary for these growth-promoting activities. It was found that exposure to volatiles from the *B. subtilis* GB03 strain did not promote growth in cytokinin receptor-deficient (*cre1*) or cytokinin/ethylene-insensitive (*ein2*) mutants. On the other hand, the *B. subtilis* GB03 volatiles did promote growth in ethylene-insensitive (*etr1*), auxin-transporter-deficient/ethylene-insensitive (*eir1*), gibberellic acid-insensitive (*gai2*), and brassinosteroid-insensitive (*cbb1*) mutants, suggesting that the promotion of growth elicited by GB03 VOCs is mediated by the cytokinin-signalling pathway (Ryu et al. 2003). Further experiments demonstrated that disease severity caused by the

necrotrophic bacterial pathogen *Erwinia carotovora* subsp. *Carotovora* was significantly decreased when *A. thaliana* seedlings were exposed to VOCs produced by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a. This phenomenon, called 'induced systemic resistance' (ISR), occurred in as little as 4 days. The exogenous application of pure 2,3-butanediol induced similar effects in a dose-dependent manner. Seedlings exposed to *Bacillus* mutants defective in 2,3-butanediol synthesis showed no disease protection, which confirmed the priming activity of this compound in ISR-induction. A set of mutant *A. thaliana* lines impaired in specific regulatory pathways, including a jasmonic acid (JA)insensitive (*coi1*), an ethylene-insensitive (*ein2*), a salicylic acid (SA)-degrading line (NahG), and a line that is SA-insensitive or non-expressor of pathogenesis-related (PR) genes (*npr1*), was then tested to identify the signalling networks necessary for ISR. Pre-exposure to VOCs from *B. subtilis* GB03 did not trigger ISR in the ethylene-insensitive line (*ein2*) and did not show pathogen resistance. In addition, to further test whether these VOCs induced known signalling pathways in *A. thaliana*, transgenic plants with b-glucuronidase (GUS) fusions to *Pr-1a* (a gene activated by SA), *Pdf1.2* (a gene activated by JA and ethylene), and *Jin14* (a gene activated by JA) were exposed to VOCs released by *B. subtilis* GB03 (Ryu et al. 2004). Of these lines, the JA/ethylene-activated *Pdf1*.2::GUS line showed increased GUS activity compared with untreated control plants.

The plants carrying an ectopic copy of the JA-activated *Jin14* gene were unaffected by *B. subtilis* GB03 VOCs; thus, ethylene signalling may be required for the activation of ISR in *A. thaliana*, independently of the SA and JA signalling pathways. Surprisingly, the VOCs of *B. amyloliquefaciens* IN937a functioned independently of all of these pathways, indicating that some VOCs utilised alternative pathways to trigger ISR (Ryu et al. 2004), and are still not known. Meanwhile, a proteomics study revealed that ethylene biosynthetic enzymes were significantly up-regulated in *A. thaliana* plants exposed to *B. subtilis* GB03 VOCs, and a transcriptomic analysis showed the up-regulation of genes related to ethylene biosynthesis (*SAM-2*, *ACS4*, *ACS12* and *ACO2*) as well as ethylene response genes (*ERF1*, *CHIB* and *GST1*) following exposure to *B. subtilis* GB03 VOCs (Kwon et al. 2010; Velivelli et al. 2015). It was demonstrated that the long-chain volatiles produced by *Paenibacillus polymyxa* E681 stimulated plant growth in *A. thaliana* and induced systemic resistance against *Pseudomonas syringae* pv. *Maculicola* ES4326 (Lee et al. 2012; Velivelli et al. 2014).

A set of hormonal mutant *A. thaliana* lines impaired in specific regulatory pathways was tested to identify the signalling networks necessary for plant growth promoting activities. Lee et al. (2012) found that exposure to volatiles from *P. polymyxa* E681 did not promote growth in cytokinin/ethylene-insensitive (*ein2*) mutants.

On the other hand, the *P. polymyxa* E681 volatiles did promote growth in jasmonic acid-insensitive line (*coi1*), a salicyclic acid-degrading line (NahG), and gibberellic acid-insensitive line (*gai2*) mutants, indicating that cytokinin/ethylene signalling is essential for the promotion of plant growth in response to *P. polymyxa* E681 volatiles. Further experiments demonstrated that the severity of the disease caused by the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *Maculicola* ES4326 was significantly decreased where *A. thaliana* seedlings were pre-exposed to VOCs produced by *P. polymyxa* E681. In addition, to further test whether *P. polymyxa* E681 VOCs induced known signalling pathways in *A. thaliana*, transgenic plants with b-glucuronidase (GUS) fusions to *Pr-1a* (a gene activated by SA), and *Pdf1.2* (a gene activated by JA and ethylene), were exposed to VOCs released by *P. polymyxa* E681. Of these lines, the SA-activated *Pr-1a::GUS* line showed increased GUS activity compared with untreated control plants; thus indicating SA signalling may be required for the activation of ISR. A further transcriptomic study of *A. thaliana* exposed to VOCs from *P. polymyxa* E681 followed by pathogen challenge revealed the induction of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling marker genes, *PR1*, *ChiB*, and *VSP2*, respectively. When the volatiles produced by *P. polymyxa* E681 were examined, it was found that this rhizobacteria produced the long-chain volatile compound tridecane. The exogenous application of

pure tridecane induced *PR1* and *VSP2* expression in a dose-dependent manner after pathogen challenge; thus indicating that SA/ET signalling is essential for the activation of ISR in response to tridecane. The researchers performed additional tests to demonstrate whether the observed growth promotion of *A. thaliana* when exposed to *P. polymyxa* E681 was correlated to carbon dioxide (CO_2). Plant growth was still enhanced when exposed to barium hydroxide ($Ba(OH)_2$) which traps CO_2, indicating that some other unknown VOCs are involved in the promotion of growth (Lee et al. 2012; Jeong et al. 2019).

In addition to the impact of BVCs, it has been speculated that increased plant growth could be due to the increase in CO_2 concentrations that is seen to rise when using the sealed petri dish method. Based on co-cultivation studies involving *Arabidopsis* and *Serratia odorifera* in a closed system, it was observed that growth promotion was closely linked to carbon dioxide enrichment. In particular, the growth of *A. thaliana* was stimulated in a closed system, where carbon dioxide was the dominant component of the volatile mixture (390–3000 ppm). By contrast, in an open system under ambient carbon dioxide concentrations, volatiles with negative effects on plant growth became dominant. It is possible that activation of the tricarboxylic acid cycle (TCA) triggers the emission of carbon dioxide, although this molecule does not accumulate to higher-than-

ambient concentrations in an open system (Kai and Piechulla 2009). Similarly, the growth of *Physcomitrella patens* (moss) was stimulated in a closed system in which carbon dioxide was the dominant component of an *S. odorifera*-derived volatile mixture, growth of this moss was inhibited in an open system due to the negative influence of volatiles (Kai and Piechulla 2010). At most, high levels of carbon dioxide have been observed to increase plant biomass by as much as 25%, although this was primarily the result of increased starch accumulation rather than biomass expansion. However, it is likely that volatiles are present at much higher concentrations in closed systems than in any found under natural circumstances (Van der Kooij et al. 1999; Ward and Strain 1999; Blom et al. 2011a).

In another study, C16 hexadecane, a long-chain hydrocarbon emitted by *P. polymyxa* E681, also protected *Arabidopsis* plants from infection by the necrotrophic pathogen *Pectobacterium carotovorum* and the hemibiotrophic pathogen *Pseudomonas syringae* pv. *Maculicola* ES4326 (Park et al. 2013). Certain volatiles produced by rhizobacteria regulate plant auxin homeostasis, and can promote growth in *Arabidopsis*. Genes for auxin biosynthesis were up-regulated when aerial parts of the plant were exposed to *B. subtilis* GB03. As observed in a transgenic (DR5:auxin-responsive reporter) *Arabidopsis* line expressing a DR5::GUS fusion, exposure to *B. subtilis* GB03

volatiles induced a decrease in auxin accumulation in leaves and an increase in roots, indicative of basipetal auxin transport activation. The decrease in auxin accumulation in leaves resulted in enhanced leaf cell elongation, whereas the increase in auxin accumulation in roots led to the development of lateral roots. Thus, despite the fact that auxin is not produced by *B. subtilis* GB03, auxin signalling must be present in the root architecture response elicited by one or more BVC produced by *B. subtilis* GB03. Auxin accumulation to the sites of synthesis was impeded by the application of the auxin transport inhibitor 1-napthylphthalamic acid, which prevented the *B. subtilis* GB03-induced reduction in shoot auxin levels and the associated growth promotion. Moreover, modifications in cell wall-loosening were observed during transcriptional analysis, which might explain the accelerated cell expansion and leaf growth associated with exposure to *B. subtilis* GB03 volatiles (Zhang et al. 2007). Further experiments revealed that exposure to *B. subtilis* GB03 volatiles caused *Arabidopsis* to increase both its photosynthetic activity and chlorophyll content.

Exposure to BVCs also enhanced endogenous sugar accumulation and also led to the partial suppression of sugar sensing in plants. In contrast to wild-type plants, enhanced photosynthetic capacity (that was not additionally increased by exposure to *B. subtilis* GB03) was observed in the two glucose-insensitive *Arabidopsis*

mutants, *gin1* and *gin2*, which lack hexokinase-dependent sugar signalling. Photosynthesis is promoted by BVCs through repression of the hexokinase-dependent sugar signalling pathway. Exposure to *B. subtilis* GB03 causes an overlap in sugar/ABA sensing in plants, as ABA-synthetic transcripts, ABA-responsive genes and ABA levels in leaves become reduced. Furthermore, the increase in photosynthetic efficiency and chlorophyll content induced by *B. subtilis* GB03 can be abolished by exogenous ABA treatment. Therefore, to enhance photosynthesis, some plants modulate endogenous sugar/ABA signalling and use soil symbionts as regulators of energy procurement by plants (Zhang et al. 2008b). Common photosynthetic markers, which are enhanced by high carbon dioxide levels, include increase of photosynthetic efficiency, chlorophyll content, and sugar accumulation (Kai and Piechulla 2009). Indeed, *Arabidopsis* exhibited enhanced photosynthetic capacity (e.g. chlorophyll content) and iron accumulation following protracted exposure to *B. subtilis* GB03 (Xie et al. 2009). Under normal growth conditions, *B. subtilis* GB03 volatiles triggered a rise in the mRNA levels of Fe-deficiency-induced transcription factor 1 (*FIT1*), as well as two of its downstream targets, ferric reductase *FRO2* and the iron transporter gene *IRT1*. However, in *Arabidopsis fit1-2* knockout mutants, volatile-induced increases in iron assimilation and photosynthetic efficiency are impaired, suggesting that volatile-induced iron assimilation is mediated by

FIT1 (Zhang et al. 2009). To stimulate iron assimilation, *Sinorhizobium meliloti* VOCs induced acidification of the *Medicago truncatula* rhizosphere, which triggers enhanced photosynthetic activity (e.g. chlorophyll content), an indicator of nutritional Fe status in plants (Carmen Orozco-Mosqueda et al. 2013). *B. subtilis* GB03 volatiles enhance salt tolerance through the sodium transporter HKT1, which is down-regulated in roots and up-regulated in shoots, resulting in a plant-wide reduction in Na+ accumulation versus control plants not exposed to GB03 volatiles (Zhang et al. 2008a). It was demonstrated that tolerance to abiotic stress could be induced in *Arabidopsis* by *Pseudomonas chlororaphis* O6 and the results suggested that this phenomenon was largely due to production of the volatile compound 2,3-butanediol. A *P. chlororaphis* O6 mutant defective in 2,3-butanediol production showed no drought resistance upon bacterial colonisation, which confirmed the role of this compound in induction of drought tolerance.

Interestingly, it was shown that 2,3-butanediol produced by this rhizobacterium facilitated both stomatal closure and drought tolerance through an ABA-1- and OST-1 kinase-dependent manner. A set of mutant *A. thaliana* lines impaired in specific regulatory pathways, including a mutant with reduced ABA synthesis (*aba1*) and a mutant deficient in the protein kinase mediating stomatal regulation in response to drought (*ost-1*)

showed no drought tolerance upon *P. chlororaphis* O6 root colonisation. When drought-stressed plants were exposed to 2,3-butanediol, the plants accumulated greater levels of SA than unexposed plants, indicating that the SA signalling pathways are involved in *P. chlororaphis* O6-induced drought tolerance (Cho et al. 2008). *Arbidopsis* plants treated with *Bacillus subtilis* FB17 significantly reduced the severity of the disease caused by the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. Tomato DC3000. This previously mentioned phenomenon, called ISR, also occurred when *Arabidopsis* plants were exposed to acetoin. *B. subtilis* FB17 mutants defective in acetoin biosynthesis showed reduced disease protection, and this result confirmed the priming activity of this compound in ISR. A set of mutant *A. thaliana* lines impaired in specific regulatory pathways, including a jasmonic acid (JA) mutant (*jar1-1*), an ethylene mutant (*etr1-3*), a salicylic acid (SA) deficient mutant (*nahG*), and a line that is SA-insensitive or non-expressor of pathogenesis-related (PR) genes (*npr1-1*), was then tested to identify the signalling networks necessary for ISR. Of these lines, treatment with *B. subtilis* FB17 and acetoin did not trigger ISR in the *etr1-3*, *nahG* and *npr1-1* lines and did not show the pathogen resistance against *Pseudomonas syringae* pv. Tomato DC3000; thus indicating that ISR elicitation is mediated via NPR1 and SA/ET signalling pathways to activate ISR in *Arabidopsis* independently of JA signalling pathway (Rudrappa et al. 2010).

In species such as *B. subtilis*, 2,3-butanediol synthesis is mediated by the transformation of pyruvate into acetocholate by the enzyme 'acetocholate synthase'. Following this, acetocholate decarboxylase converts alpha-acetocholate into acetoin which is subsequently converted to 2,3-butanediol via catalysis mediated by the acetoin reductase/2,3-butanediol dehydrogenase (AR/BDH) (Nicholson 2008).

The conversion of glucose into 2,3-butanediol and acetoin occurs under hypoxic conditions and serves as an electron sink for the generation of NAD+ when aerobic respiration is restricted. Furthermore, low partial oxygen pressure (as generally exists in soil surrounding roots) induces the bacterial acetoin pathway that controls the production of 2,3-butanediol. Consequently, it is likely that 2,3-butanediol and/ or other biologically active molecules are produced by certain root-colonising rhizobacteria at concentrations appropriate to elicit plant reactions (Ryu et al. 2003, 2004). The Methyl Red Voges Proskauer (MR-VP) medium is generally used to determine the ability of bacteria to ferment 2,3-butanediol (Nicholson 2008). As a by-product of the fermentation pathway employed by some rhizobacteria to avoid acidification, the biosynthesis of 2,3-butanediol is often induced on low pH Murashige and Skoog (M+S) medium containing sucrose (Ryu et al. 2003). The growth of *Penicillium* spp. Was suppressed both in vitro and in vivo by volatiles released by

Bacillus spp., and citrus fruit inoculated with *Penicillium crustosum* showed reduced disease incidence and severity due to the presence of acetoin. Furthermore, it was observed that longer exposure times led to stronger volatile-mediated antifungal effects; this was attributed to the extended incubation period within the closed system, leading to the restriction of oxygen over time (Arrebola et al. 2010).

On the other hand, it was also demonstrated that the acetoin pathway is optimal in the lifecycle of the necrotrophic bacterial pathogen *Pectobacterium carotovorum* subsp. *Carotovorum* WPP14. Mutants defective in the 2,3-butanediol pathway were unable to alkalinise growth media and also showed reduced virulence on potato tubers (Marquez-Villavicencio et al. 2011). Furthermore, the capacity of *Bacillus megaterium* XTBG34 to promote growth in *A. thaliana* was validated by Zou and Co-workers. In particular, a number of compounds produced by this organism, including 2-pentylfuran, were identified by GC/MS analysis, and they showed that plant growth was significantly enhanced by 2-pentylfuran in a dose-dependent manner. The lowest dose at which this compound could enhance plant growth was 0.1 µg, and maximum growth was achieved with 10 µg; by contrast, doses greater than 10 µg inhibited growth (Zou et al. 2010). Santoro and Co-workers examined the effects of BVCs on growth promotion and the enhanced biosynthesis of essential oils

such as pulegone and menthone in *Mentha piperita* (peppermint). The results of this study indicated that BVCs exhibit species-specific effects on plants. BVCs not only trigger secondary metabolite production but also impact pathway flux during certain stages of monoterpene metabolism (Santoro et al. 2011). It was demonstrated that volatiles released by *Proteus vulgaris* JBLS202 stimulate growth in Chinese cabbage and GC/MS analysis showed that indole was the primary headspace volatile compound produced by this bacteria. They showed that plant growth was significantly enhanced by indole in a dose-dependent manner. When plants were exposed to 0.63 µg of synthetic indole, growth was significantly enhanced. Indole and its derivatives are known to be involved in the auxin signalling pathway (Yu and Lee 2013).

Blom and colleagues analysed the effects of different bacterial strains cultured on four distinct media on the growth of *A. thaliana*. Of the bacterial strains tested, one strain promoted growth on all four media tested. GC/MS analysis revealed the presence of a range of compounds, including indole, 1-hexanol and pentadecane, and they showed that plant growth was affected in a concentration-dependent manner by these compounds. Indole promoted growth at low concentrations but showed lethal effects when used at high concentrations. Furthermore, when 1-hexanol was applied in moderate amounts, it showed weak growth

promotion, and pentadecane promoted growth when applied at high concentrations (Blom et al. 2011a).

In another study, rhizobacterial strains were isolated from the rhizosphere of lemon plants (*Citrus aurantifolia*) and then analysed to determine whether their VOCs had an effect on the development of *A. thaliana* roots. Using a simple experimental system involving I-plates, the authors observed several morphological changes in root architecture due to VOCs. It is interesting to note that some rhizobacterial strains stimulated primary root growth and lateral root development. Several compounds were detected by GC/MS, including aldehydes, ketones and alcohols. However, short-chain alcohols, such as 2,3-butanediol and acetoin were not identified in this study, indicating that other VOCs can also trigger plant growth (Gutiérrez-Luna et al. 2010). Exposure of *A. thaliana* plants with *Bacillus megaterium* UMCV1 modified the architecture of the root system in this plant. In particular, the authors observed an inhibition of primary root growth as well as increases in lateral root number, lateral root growth, and root hair length, and they found that reduced cell elongation and cell proliferation in the root meristem was the cause of the inhibition of primary root growth. The analysis of *Arabidopsis* mutant lines defective in either ethylene (*etr1* and *ein2*) or auxin (*aux1-7*, *axr4*, *eir1*) signalling revealed that, modifications in root architecture caused by *B. megaterium*

UMCV1 may involve either auxin- or ethylene-independent mechanisms. Furthermore, transgenic *Arabidopsis* line expressing a DR5::uidA (a reporter line for auxin and ethylene-inducible gene expression) GUS fusion showed reduced expression in root tips (López-Bucio et al. 2007).

Velázquez-Becerra and Co-workers tested the effects of *Arthrobacter agilis* UMCV2 volatiles on alfalfa (*Medicago sativa*), and they found that *A. agilis* volatiles decreased taproot growth and increased lateral root formation, indicating that the BVCs emitted by this bacterium play an important role in root development. Analysis of BVCs produced by this organism revealed a range of compounds, at least one of which, N-N-dimethyl-hexadecanamine, may act as a growth-promoting trigger, affecting root development in *Medicago sativa* in a dose-dependent manner (Velázquez-Becerra et al. 2011).

A study by Tahir and colleagues determined the effect of BVCs from *Bacillus subtilis* SYST2 on tomato. Two compounds, albuterol and 1,3-propanediol were identified as having a positive effect on plant growth with observed increases in auxin and cytokinin in the plant tissues and noticeable increases in expansin gene transcripts. Variations in VOC concentrations and/or plant exposure times can dictate whether an inoculation has positive or negative effects on primary root growth and/or lateral root

formation (Tahir et al. 2017b). *Arabidopsis* plants exposed to *Burkholderia ambifaria* volatiles show enhanced biomass, greater numbers of secondary roots and shorter main roots. Analysis of the VOCs produced by this bacterium revealed a range of compounds, including dimethyl disulphide (DMDS), acetophenone and 3-hexanone, 4-methyl-2-pentanone, 4-octanone, and 2,5-dimethylpyrazine. These compounds were shown to affect plant growth in a concentration-dependent manner, and indeed, treatment with very high amounts could inhibit plant growth. Plants showed greater biomass when exposed to some concentrations of DMDS, acetophenone and 3-hexanone. By contrast, high concentrations of 4-methyl-2-pentanone and 4-octanone were lethal to the plants. Finally, 2,5-dimethyl pyrazine promoted growth at lower concentrations, whereas higher concentrations were deleterious to *Arabidopsis* plants (Groenhagen et al. 2013).

Many studies have shown that BVC interact with the host root system and in addition to its role as a structural support for the plant, it is also crucial for the acquisition of water and nutrients from the soil. From an ecological perspective, BVC-mediated alterations in the root system proteome and root architecture may have beneficial effects through increasing bacterial root colonisation and optimising symbiotic interactions (Yaoyao et al. 2017). These symbiotic relationships mediate biochemical

interactions which stimulate root growth and development which leads to enhanced levels of bioavailable nutrients for the plant (Velivelli et al. 2015; Hérnandez-Calderón et al. 2018). In reciprocation, PGPR gain access to richer sources of nutrition and carbon through root exudates produced by a healthy plant host (Gutiérrez-Luna et al. 2010; Velivelli et al. 2015).

Inhibitory Effects of Rhizobacterial Volatiles on Plants:

In addition to promoting plant growth, it has been demonstrated that certain BVC have negative effects on plants such as *A. thaliana* (Vespermann et al. 2007). One of the most important sources of nitrogen is ammonia, although this compound has recently been shown to play a number of other biological roles. When *S. odorifera* 4Rx13 is grown on a peptone-rich medium, it produces high levels of ammonia, and when this BVC was exposed to *A. thaliana* plants in an I-plate, the bacterium caused the neighbouring plant medium to become alkalinised, leading to reduced plant growth (Weise et al. 2013). Under conditions of low oxygen, such as in a closed system, the production of HCN by some *Pseudomonas* sp. is enhanced (Athukorala et al. 2010).

Deleterious effects were observed when *A. thaliana* was exposed to HCN, with a four-fold reduction in growth following exposure to 1 µmol HCN (Blom et al. 2011b). In has also been shown that rhizobacterial volatile compounds such as ammonia and DMDS have negative effects in higher concentrations on *A. thaliana* growth (Kai et al. 2010).

Effect of Rhizobacterial Volatiles on Fungi and Other Organisms:

For truly sustainable agriculture, the strategies we employ to combat plant diseases must become more environmentally-friendly with lower inputs of synthetic chemicals. The use of beneficial microbes as a biological input to sustainable agricultural systems offers an alternative, and potentially more environmentally stable approach, to conventional agri-chemical-based solutions for the suppression of plant pathogens and the treatment of plant diseases in an integrated pest management system (Velivelli et al. 2015). Despite their powerful antifungal activities, non-volatile antibiotics are unable to spread over long distances, making them only effective at preventing infection by pathogenic microbes/fungi when applied directly to plant roots.

The ability of BVCs to suppress the growth and proliferation of plant pathogens has attracted ample attention with regard to biological applications and rhizobacterial VOCs have displayed antagonistic activity against pathogenic fungi, which may classify them as novel antibiotic compounds (Velivelli et al. 2015). The best known example of one such inorganic volatile metabolite is hydrogen cyanide.

Hydrogen cyanide (HCN) is a secondary metabolite produced by some gram-negative *Pseudomonas* spp. Upon the hydrolysis of glycine by HCN synthase. *Pseudomonas fluorescens* CHA0 was shown to inhibit the development of *Thielaviopsis basicola*, which causes black root rot in tobacco plants, through the production of HCN (Bailly and Weisskopf 2017). The biocontrol potential of these antibiotics has been experimentally validated through the use of mutant rhizobacterial strains with altered antibiotic production. A hydrogen cyanide negative mutant (*hcn*), *P. fluorescens* CHA0 strain was no longer able to protect tobacco against black root rot (Voisard et al. 1989; Blumer and Haas 2000). A more recent study (Rijavec and Lapanje, 2016) proposed that the main contribution of HCN to biocontrol is more indirect and is related to the sequestration of metals and the associated beneficial increase of nutrients to the plant and rhizobacteria.

The volatile inorganic compound ammonia, which is released by the rhizobacteria *Enterobacter cloacae*, suppressed the growth of *Pythium ultimum* in dual-culture assays, thus describing its possible role in the biological control of *Pythium* pre-emergence damping-off (Howell et al. 1988). In addition to HCN and ammonia, the antifungal nature of the organic volatiles has been demonstrated in several experiments. *Pseudomonas* spp. Isolated from canola and soybean plants were reported to produce volatile antibiotics including; n-decanal, nonanal, 2-ethyl-1-hexanol, benzothiazole and dimethyl trisulfide, that inhibit the fungal pathogen *Sclerotinia sclerotiorum* in I-plate assays (Fernando et al. 2005). Furthermore, the growth of *S. sclerotiorum* was also suppressed in antifungal bioassays performed in sealed plates containing pure synthetic volatiles such as furfural, benzaldehyde, 1-octanol, 1-octen-3-ol, 3,7-dimethyl-1-ol, 6-octadien-3-ol and 2-ethyl-1-hexanol (Liu et al. 2009).

The growth of *Fusarium oxysporum* f. sp. *cubense* was suppressed in an I-plate assay by BVCs produced by *Bacillus amyloliquefaciens* NJN-6. The volatile organic compounds emitted by this organism were diverse and included; benzothiazole, phenol, 2,3,6-trimethyl-phenol, 2-ethyl-1-hexanol, 2-undecanol, 2-nonanone, 2-decanone, nonanal, naphthalene, 2-methyl naphthalene and 1-methyl naphthalene. The application of pure, synthetic volatiles in the same bioassay revealed strong

antifungal activities against *F. oxysporum* f. sp. *cubense* (Yuan et al. 2012).

Jasmonic acid BVCs produced by *B. subtilis* significantly inhibited the spore germination of *Botrytis cinerea* in an I-plate assay. An analysis of VOCs revealed a range of compounds, such as 4-hydroxybenzaldehyde, 2-nonanone, ammonium acetate, 1,2,4,5-tetramethyl-pyrazine, 9-methyl-nonadecane, 2,6,11,15-tetramethylhexadecane, 2,6,10,15-trimethyl-tetradecane and 8-hexyl-pentadecane; however, the authors did not evaluate the antagonistic potential of these compounds against *Botrytis cinerea* (Chen et al. 2008). The BVC 2-nonanone showed an inhibitory effect towards *B. cinerea* fungal decay of strawberries in closed containers, thus suggesting its potential role in reducing post-harvest diseases of agricultural products, an area in which there is a significant increase in research activity worldwide (Almenar et al. 2007; Sharifi and Ryu 2018b).

The growth of the soil-borne pathogenic fungi *Rhizoctonia solani* was strongly inhibited in an I-plate assay by BVCs emitted by a number of common soil bacterial genera such as *Bacillus* spp., *Pseudomonas* spp., *Serratia* spp., and *Stenotrophomonas* spp. Further molecular analysis revealed a wide array of compounds including: b-phenylethanol, trans-9-hexadecene-1-ol, undecene, undecadiene, dodecanal, benzylnitrile, benzyloxybenzonitrile,

and dimethyl trisulfide (Kai et al. 2007). Dimethyl disulphide was shown to inhibit the growth of *Fusarium culmorum* in a dual-culture assay and this inhibitory effect on mycelial growth was observed to occur in a dose-dependent manner; less obvious effects were also observed with the use of pure 1-undecene (Kai et al. 2009). Antifungal volatile metabolites produced by *A. agilis* UMCV2 inhibited the growth of *B. cinerea* on sealed plates. The volatile organic compound, dimethylhexadecylamine, which is released by the rhizobacteria *A. agilis*, inhibited the growth of both *B. cinerea* and *P. cinnamomi* in dual-culture assays when provided to the culture medium at low concentrations (Velázquez-Becerra et al. 2013). It has been demonstrated that BVCs produced by *Streptomyces plantesis* F-1 could inhibit the growth of *R. solani*, *B. cinerea* and *S. sclerotiorum*. Exposure to *S. plantesis* F-1 BVCs significantly reduced the incidence and severity of leaf blight/seedling blight caused by *R. solani*, leaf blight of oilseed rape caused by *S. sclerotiorum* and fruit rot of strawberry caused by *B. cinerea*; thus indicating its possible role as a biofumigant in the biological control of fungal diseases. The analysis of volatile organic compounds from this organism revealed diverse compounds, including but not limited to phenylethyl alcohol, phenol, 2,5-bis(1,1-dimethylethyl), (+)epi-bicyclesesquiphellandrene and cyclohexane carboxylic acid; however, the potential role of these compounds remains to be investigated. As suggested by the complex nature of BVCs,

significant growth inhibition may require the synergistic activity of multiple compounds or the activity of extremely potent infochemicals normally present at low concentrations (Wan et al. 2008). It has been demonstrated that the volatile metabolites produced by fungistatic soils suppress the growth of *Paecilomyces lilacinus*, *Pochonia chlamydosporia* and *Clonostachys rosea*. In particular, VOCs produced by fungistatic soils, including trimethylamine, benzaldehyde, and N,N-dimethyloctylamine elicit strong antifungal activity in a sealed petri plate assay containing known amounts of fungal spore suspension, autoclaved soil and/or pure synthetic compounds (Chuankun et al. 2004).

In an I-plate assay, the volatile organic compound, O-anisaldehyde, which is released by *Bacillus atrophaeus* CAB-1 inhibited the growth of *B. cinerea*; thus suggesting its possible role in soil fungistatis and the subject of further study (Zhang et al. 2013). The pathogenic activity of various fungal pathogens was suppressed by volatiles emitted by *P. polymyxa* strain BMP-11. The mycelial growth of various fungal pathogens, including *R. solani*, was suppressed in sealed Petri dish antifungal bioassays involving pure, synthetic volatiles such as 1-octen-3-ol, benzothiazole and citronellol; mycelial morphological deformities were also observed (Zhao et al. 2011). Antifungal metabolites produced by *B. subtilis* inhibited the growth of two

strains of *R. solani* in sealed I-plate assays. Interestingly, these two fungal strains reacted differently to exposure to the *B. subtilis* BVC mixtures, indicating that BVC-mediated interactions between bacteria and fungi can be species/genus specific. Therefore, it is possible that complex volatile mixtures may trigger significantly different responses in different fungi, which may for example be due to differentially conserved molecular activities of fungi for detoxifying metabolites.

Fungal growth modifications due to BVC exposure are not uncommon and indeed are closely linked to growth medium and inoculum dose, and in soil, volatile emissions have been linked to nutrient availability, pH, temperature and oxygen availability (Schulz-Bohm 2017). Rhizobacterial strains emit volatiles that can have distinct effects on fungi or inhibit different fungal types to varying degrees. This could be due to the fact that different BVC blends are released by various rhizobacteria, and inhibitory effects may be induced due to the synergistic effects of several compounds within those respective blends (Velivelli et al. 2015). For example, the fungistatic BVC emitted by *Bacillus cereus* were shown to more strongly repress a *Trichoderma viride* strain than a *Gelasinospora cerealis* strain. Similarly, the BVCs released by a strain of *Aerobacter aerogenes* were not as effective against *F. oxysporum* f. sp. *conglutinans* as they were against *T. viride* and *Penicillium* sp. (Fiddaman and Rossall 1994).

Using an I-plate assay, it was shown that exposure to *Burkholderia ambifaria* VOCs reduced the growth of fungi, and this inhibitory effect was observed to be stronger against *Alterneria 92on-patho* than it was for *R. solani*. In particular, the growth of *A. 92on-patho* and *R. solani* was decreased by higher doses of dimethyl trisulphide, 2-nonanone, 1-phenyl-1,2-propanedione, and 2-undecanone. Moreover, *R. solani* was also suppressed by acetophenone, phenylpropan-1-one, DMDS, 4-octanone and S-methyl methanthiosulphonate. The growth of *Fusarium solani* was not reduced by any of the volatiles tested, indicating that fungi react to BVCs differently (Groenhagen et al. 2013; Velivelli et al. 2015).

Bacterial volatile patterns can also be affected by growth media. For example, BVC emission profiles differed based on whether the media (nutrient broth) they were grown on contained glucose. The growth of *R. solani* on nutrient broth (NB) was more strongly suppressed by the volatiles produced from *Xanthomonas campestris* pv. *Vesicatoria* 85-10 when the latter was grown on NB than when it was grown on nutrient broth with glucose (NBG). *A. nidulans* and *F. solani* exhibited similar growth patterns. The inhibition of *A. nidulans* was stronger when *X. campestris* pv. *Vesicatoria* 85-10 was grown on NB than when it was grown on NBG. When *X. campestris* pv. *Vesicatoria* 85-10 was grown on NB, the growth of *A. nidulans* and *F. solani* was inhibited by 85%

and 14%, respectively, whereas growth inhibition on NBG was only 11% and 3.5%, respectively. The BVCs of *X. campestris* pv. *Vesicatoria* 85-10 showed only weak effects on *F. solani*, indicating species-specific activity. These results demonstrate that when grown on glucose-containing media, *X. campestris* pv. *Vesicatoria* 85-10 BVCs have only a weak effect on fungi, indicating that nutrient levels influence growth inhibition. There are several potential explanations for the observed reduction in growth suppression by *X. campestris* pv. *Vesicatoria* when grown on NBG: (1) growth on NB media stimulates the production of a larger amount of suppressive volatiles; (2) the production of suppressive volatiles relies on peptone-rich NB media; (3) the production of suppressive volatiles is restricted by glucose through a mechanism such as catabolite repression; or (4) there is a delay in the production of suppressive volatiles on NBG media (Weise et al. 2012). Fiddaman and Rossall (1994) observed that *B. subtilis* only produced suppressive BVCs in the presence of D-glucose, and not L-glucose (Fiddaman and Rossall 1994). Previously, Fiddaman and Rossall (1993) had assumed that agar containing high levels of glucose, i.e. PDA (potato dextrose agar) and SGA (Sabouraud's glucose agar) with *B. subtilis* would stimulate significant antifungal activity in vitro. By contrast, limited or no in vitro antifungal activity was produced by agar containing little or no glucose (VJA (V8 juice agar), NA (nutrient

agar) or 10% TSA (tryptic soy agar) (Fiddaman and Rossall 1993).

Serratia plymuthica strains emitting DMDS as the primary headspace BVC, were seen to inhibit the growth of *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains in dual-culture assays. When *Solanum lycopersicum* plants were inoculated with *S. plymuthica*, it inhibited the growth of *Agrobacterium*, and resulted in the emission of DMDS by the *S. lycopersicum* plants (Dandurishvili et al. 2011). It was demonstrated that *S. plymuthica* HRO-C48 inhibited the growth of *R. solani* using I-plates (Kai et al. 2007). When oilseed rape cv. Talent was treated with *S. plymuthica* HRO-C48, disease severity of *Verticillium 94on-pa* was significantly reduced. This strain also produces DMDS and is registered and distributed by RhizoStar®, E-nema GmbH Raisdorf, Germany (Müller et al. 2009). This strain was shown to suppress *V. 94on-pa* in strawberry and *R. solani* in lettuce (Kurze et al. 2001; Grosch et al. 2005). The development of DMDS has been targeted as a possible alternative to the fumigant methyl bromide. DMDS was observed to supress phytopathogenic nematodes and fungi in the soil, offering strong evidence for the effect of DMDS against plant-pathogenic fungi (Fritsch 2005) and nematodes (Coosemans 2005). Based on these findings, rhizobacteria that produce DMDS can be classified as natural fumigants and this biocontrol capacity is closely

associated with VOC production. Therefore, antifungal VOCs should be considered as significant tools for the control of plant pathogens, in addition to more conventional agrichemicals. At present, Paladin®, a new and effective soil fumigant based on DMDS, had already been registered in the USA, (http://www.arkema.com/en/media/news/news-details/Arkema-receives-U.S. –registration-for-Paladin-soil-fumigant-00001/) and the development of new soil fumigants based on DMDS is progressing in Europe.

The growth of *F. oxysporum* was suppressed in an I-plate assay by the BVCs produced by endophytic bacteria. A range of compounds was observed during the analysis of these volatiles, such as 2-pentanone,3-methyl, methanethiol and 3-undecene. However, the authors did not evaluate the antifungal properties of the individual compounds on *F. oxysporum* (Ting et al. 2011). It has been demonstrated that the volatile metabolites produced by endophytic *Burkholderia tropics* strains inhibited the growth of four phytopathogenic fungi: *Colletotrichum gloeosporioides*, *Fusarium culmorum*, *F. oxysporum* and *Sclerotium rolfsii*. Further analysis revealed the existence of numerous compounds, including a-pinene, ocimene, limonene and fencona, indicating that these compounds may also play important antifungal roles (Tenorio-Salgado et al. 2013). Considering the effects of BVC on the development of fungi and plants, an obvious question is

whether these effects extend to animals living within the soil as well.

In an I-plate assay, volatiles released by *Bacillus megaterium* YMF3.25 were shown to inhibit the hatching of nematode eggs and reduce nematode infections. GC/MS analysis revealed a variety of compounds, such as benzeneacetaldehyde, 2-nonanone, decanal, 2-undecanone and DMDS that exhibited strong nematicidal activities against both juveniles and eggs. Additional research will be required to create integrated management systems for lowering root-knot nematode inocula and enhancing crop yields in the field, perhaps by improving bacterial formulation. (Huang et al. 2010).

An analysis of *Lysinibacillus mangiferahumi* BVCs showed nematicidal activity against the root-knot nematode *Meloidogyne incognita*. Volatile analysis revealed the presence of numerous compounds, including 2-octanol, cyclohexene, 3-chloro-4-fluorobenzaldehyde, dibutyl phthalate, 2-nitro-2-chloropropane, dimethachlore, and DMDS. When nematodes were exposed to these pure volatiles in a three-compartment Petri dish assay, the compounds showed growth-inhibitory effects towards nematodes (Yang et al. 2012). Similarly, BVCs produced by soil bacteria showed nematicidal activity against *Panagrellus redivivus* and *Bursaphelenchus xylophilus* in three-compartment Petri dish

bioassays. Further analysis revealed significant difference in the nematicidal activity of the VOCs, indicating that VOC-mediated interactions between bacteria and nematodes can be species-specific/isolate-specific. Numerous distinct volatiles were identified, including phenol, octanol, benzaldehyde, benzene, acetaldehyde, decanal, 2-nonanone, 2-decanone, cyclohexene, and DMDS. Finally, exposure to pure synthetic VOCs using the same bioassay revealed growth-inhibitory activities against both *B. xylophilus* and *P. redivivus* (Gu et al. 2007).

In a recent study by Cho et al. (2017) it was observed that a novel antifungal volatile, caryolan-1-ol, produced by *Streptomyces* spp., was effective at inhibiting the growth of the fungal pathogen *B. cinerea*. By using a homozygous profiling (HOP) assay in *Saccharomyces cerevisiae,* in which both copies of non-essential genes are deleted, it was determined that caryolan-1-ol most likely inhibits fungal growth by targeting membrane lipid processes and intracellular transport systems in fungi (Cho et al. 2017)

For many bacterial phytopathogens an integral component of their ability to infect susceptible plant hosts is the capacity to be motile, in order to actively seek out sites of colonisation and subsequent infection. To this end, Tahir and colleagues investigated the potential of BVCs from six *Bacillus* spp., to inhibit the motility of the bacterial phytopathogen *Ralstonia solanacaerum* (Rsc)

TBBS1, the causative agent of bacterial wilt disease in tobacco. They observed a particularly strong inhibitive effect of BVCs from two strains; *Bacillus amyloliquefaciens* FZB42 and *Bacillus artrophaeus* LSSC22. Three BVCs; 1,2-benzisothiazol-3(2 H)-one, benzaldehyde and 1,3-butadiene all negatively influenced cell viability, colony size, motility and chemotaxis. Transcriptomic investigation of this activity identified alterations in the expression of pathogenesis-related genes such as *PhcA* which is a global regulator of virulence factors in Rsc, as well as genes involved in type III and IV secretion systems, production of extracellular polysaccharides and chemotaxis. Defence genes in tobacco were also up-regulated with over-expression of the proteins EDS1 and NPR1 suggesting the activation of the SA pathway in the ISR response to Rsc-challenge (Tahir et al. 2017c). Interestingly, in a separate study by Tahir and colleagues it was observed that the BVCs produced by Rsc could not elicit a significant plant growth promoting effect. However they did inhibit the growth-promoting potential of *B. subtilis* SYST2 BVCs when plants were exposed to BVCs emitted by both SYST2 and Rsc. Co-culture of both bacteria together revealed that they inhibited the growth of one another, but the effect of inhibition by Rsc of SYST2 was not as great as SYST2 versus Rsc (Tahir et al. 2017c)

Species-Specific Rhizobacterial Volatiles:

The qualitative and quantitative complexity of the VOC profiles of various rhizobacteria can vary significantly. For example, common rhizobacterial strains such as *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, release a mixture of volatile compounds (e.g. 2,3-butanediol and acetoin) that promote growth in *Arabidopsis thaliana*, whereas other rhizobacterial strains that do not promote growth via VOCs produce different mixtures of compounds, indicating that synthesis of VOCs is a strain-specific phenomenon. Studies have shown that whereas some compounds are isolate-specific, others are produced by more than one type of bacteria (Ryu et al. 2003; Kai et al. 2007). Furthermore, BVC profiles may be affected by growth phase and environmental conditions. For example, different qualitative and quantitative VOC profiles are generated by growth on nutrient broths with and without glucose in *Stenotrophomonas rhizophila* P69. In particular, the production of dimethyl pyrazine and beta-phenylethanol occurs under both growth conditions, whereas trimethyl pyrazine, tetramethyl-pyrazine and beta-phenylethyl acetate are produced when glucose is absent from the growth medium (Kai et al. 2009).

The effect of different bacterial strains cultured on four distinct types of media on the growth of *A. thaliana* were analysed by

Blom and co-workers. They found that more nutrient-rich media Luria-Bertani (LB) caused plant death, whereas all other media tested—including two less nutrient-rich media, Murashige and Skoog (M+S) and the soil mimicking Angle medium—promoted growth (Blom et al. 2011a). M+S and LB have significantly different compositions; M+S is a mineral medium that contains sugar as a carbon source and has low pH and agar concentrations, whereas LB is a nutrient-rich medium containing hydrolysed proteins with relatively high pH and agar concentrations. Therefore, it is perhaps unsurprising that the same bacteria cultured on these two media types can emit different types of BVCs that elicit different plant responses. Furthermore, these two media types also affect the growth kinetics (maximal volatile production, which is assumed to take place during the stationary phase of bacterial growth) of bacterial strain on these two media, with LB supporting faster growth than M+S (Bailly and Weisskopf 2012). The primary factors that determine the qualitative and quantitative distribution of compounds in highly complex mixtures are the metabolic abilities of the bacterial species, as well as the nutrients available in the specific growth conditions. Indeed, differences in the types of BVCs produced by pathogenic and non-pathogenic mycobacteria grown on different types of media have been observed; furthermore, variations were sometimes observed, not only between different media but also within individual analyses (Nawrath et al. 2012). However, it is

important to note that results obtained under artificial conditions may not accurately reflect natural conditions and an in-depth understanding of this area of plant-microbe research cannot be acquired solely from in vitro experiments of VOC-mediated interactions between all organisms involved in these interactions. Results obtained in controlled, artificial environments may not, and indeed most likely do not, represent interactions which would be observed in the field (Velivelli et al. 2015).

This issue of the interpretation of results from field versus lab is relevant for metabolic experiments utilising artificial growth media and nutrient sources and also for interactions where BVCs are outside of a detectable range. Such concentrations of BVCs observed under in vitro conditions are unlikely to be naturally present in the soil. Nevertheless, in vitro experiments contribute greatly to our understanding of the molecular interplay between bacterial volatiles and other organisms. This can give an insight as to what may be observed within these complex relationships in the field (Ryu et al. 2003; Blom et al. 2011a; Effmert et al. 2012; Park et al. 2015; Besset-Manzoni et al. 2017; Brilli et al. 2019; Song et al. 2019). Furthermore, not all plant species respond similarly to the same group of volatiles produced by a given bacterial strain, which could be due to several reasons: (1) fundamental differences in the pathways used by plants to respond to BVCs (2) differences in reactive sites and (3) differences in the

capacity to metabolise volatiles (Santoro et al. 2011). The volatiles produced by a given bacterial strain elicited different responses in different fungi; in other words, differences exist in the interactions between different fungi and bacteria. For example, although *Arabidopsis* growth was significantly promoted by DMDS; the responses of different fungal species to this compound varied considerably: the growth of *R. solani* was suppressed, whereas *A. alternata* and *F. solani* were unaffected (Groenhagen et al. 2013). Therefore, it is necessary to employ a number of different approaches and to test bacteria under distinct growth conditions to determine comprehensive bacterial emission patterns (Farag et al. 2017).

Analytical Approaches to Identify VOCs:

The capture of VOCs which are emitted as a result of microbial metabolic activity is the first and most crucial step in the analysis of biological VOCs, and research in this area of 'separation science' has advanced significantly in the last half a century (Velivelli et al. 2015). SPME-GC/MS and PTR-MS are just two of the numerous approaches designed for the capture and subsequent identification of volatiles. Although each approach has its own advantages, no single method is currently capable of completely surveying bacterial-produced volatile profiles, either in terms of quantity or quality. To successfully identify volatiles,

a number of sampling techniques are generally employed, such as purge and trap, solid phase microextraction (SPME) followed by gas chromatography/mass spectrometry (GC/MS) identification. The purge-and-trap method involves passing a given volume of purified air over the sample, collecting it onto an absorbent filter and then either directly releasing it using an organic solvent to rinse the filter or transferring it straight to the GC/MS (Ryu et al. 2003, 2004; Yuan et al. 2012). Solid phase microextraction (SPME) has become the method of choice for the extraction of bacterial volatiles in recent years because it minimises preparation time and has greater sensitivity than other extraction techniques.

The process of SPME for the analysis of bacterial volatiles is relatively fast and can be conducted under low oxygen conditions. Although SPME has many advantages over other methods, it is necessary to carefully consider the particular fibre coatings used in each experiment, as they can either absorb or exclude specific analytes based on polarity or size, leading to reduced sensitivity. For instance, non-polar metabolites are absorbed by Polydimethylsiloxane (PDMS) fibre, whereas short-chain polar compounds are absorbed by divinylbenzene/carboxen/ PDMS (DCP) fibre. With respect to rhizobacterial volatiles, the best recovery is offered by DCP fibre, as it absorbs polar low molecular weight volatiles (Farag et al. 2006). Yuan and Co-workers tested three different fibres from Supelco—PDMS, 7 µm,

stable flex DCP, 50/30 µm, and polydimethylsiloxane/divinylbenzene (PDMS-DVB, 65 µm)—and they found that DCP fibre performed best (Yuan et al. 2012). The volatile extraction methods discussed above are often used in combination with other analytical techniques, such as GC/MS. Considering its effectiveness for both separation and identification, GC/MS is the foremost method for the detection of bacterial volatiles. GC/MS can be used to separate, identify and quantify the volatiles present in a given sample. However, this technique has one significant drawback; namely, it does not allow for the identification of new compounds. Furthermore it can be difficult to obtain quantitative results using SPME, as volatile compounds compete for binding sites within the SPME fibre. Small bacterial molecules are characterised by high polarity and a strong tendency to co-elute. As a result, overlapping MS spectra can be produced, limiting the precision of volatile detection and adversely affecting the process of matching peak quality against database entries.

The identification of existing volatiles is carried out using software programs and libraries of different mass spectra such as NIST/or reference standards. Nevertheless, as similar mass spectra can be produced by related compounds, particular care must be taken in performing these analyses (Kai et al. 2009). A recently developed technique with the advantage of real-time

analysis without requiring sample preparation is PTR-MS. In proton transfer reaction-mass spectrometry (PTR-MS), the headspace air is drawn directly into the instrument, where interactions occur between protonated water (H_3O^+) and any molecules with a proton affinity greater than that of water. Next, a quadruple mass spectrometer in mass-to-charge (m/z) ratio is used to mass analyse and identify the resulting protonated organic molecules. Although PTR-MS cannot accurately detect the individual volatiles produced, it is advantageous because it ensures real-time emission observation.

In addition, PTR-MS cannot be used to distinguish between analytes of the same mass because it employs single reagent ions (Spinelli et al. 2012). PTR-MS was used to determine the volatile profiles of various bacteria and infected plants, and it allowed for the observation of pathogenic emissions in real time (Spinelli et al. 2011). Several researchers have analysed the volatiles emitted by bacteria, and a comparison of these signature compounds indicates that GC/MS analysis can be used to distinguish between different bacterial species. Indeed, the VOC-profile data obtained from GC/MS could be harnessed to develop so-called 'electronic-nose' (e-nose) sensor technology for the detection of different bacterial species, or they may be useful for real-time disease monitoring in agricultural settings and post-harvest monitoring where e-nose technology could detect ring and brown rot in

commercial potato storage with a detection efficiency of 90% of samples (Biondi et al. 2014). Currently, the identification of bacterial species, including phytopathogenic bacterial isolates, is achieved through 'classical' molecular techniques such as polymerase chain reaction followed by dideoxy sequencing of 16S rRNA and these methods, although reliable, are expensive (Velivelli et al. 2015). The efficacy of e-nose technology has been demonstrated for the detection of a single phytopathogen within a wider microbial community; using a metal-oxide-based semiconductor sensor the pathogen that causes fire blight (*E. amylovora*)—could be differentiated based on volatile mixes alone from other bacteria (Spinelli et al. 2012). However, this technique has one significant drawback; it does not identify and quantify each compound; rather, it uses metal-oxide semiconductor sensors to characterise an aroma based on the signature profile that is generated.

The e-nose distinguishes between *Botrytis*- and *Sclerotinia*-induced rots on both green and yellow kiwifruits. In addition, it was successfully used to detect asymptomatic apple and pear plants that had been infected with *Erwinia amylovora*. More research is needed before e-nose applications in open fields becomes standard practice (Spinelli et al. 2010). In 2015, Cernava et al. described a novel assay for the detection of bacterial volatiles from lichen-associated bacteria using headspace analysis

and indicator assays (for HCN production) followed by the determination of the effect of the VOCs on the growth of *E. coli* and *B. cinerea*. The initial test was combined with a qPCR-based quantification assay (Cernava et al. 2015).

Discussion and Conclusive Remarks:

Research into volatile-mediated plant–bacteria interactions is relatively new, and this field has the potential to yield significant agricultural applications, particularly in the context of growth promotion and ISR (Liu and Brettell 2019; Romera et al. 2019). This niche of plant-bacteria research is still emerging and at present, the biological effects of BVCs have only been assessed in a relatively small cohort of plant species with *Arabidopsis thaliana* being one of the main candidates, along with more economically relevant plants such as *Capsicum annum* and *Nicotiana tabacum* (Choi et al. 2014; Kim et al. 2015). How these bacterial volatiles will affect the growth and disease-suppression in other major crops remains to be seen. In general, I-plates are

used to perform experiments on the effects of bacterial volatiles on plants and fungi. This setup allows for the exchange of volatile compounds while preventing non-volatile metabolites from dispersing throughout the respective growth media. However, following identification of the bio-active compounds, further in vitro laboratory tests should be conducted under conditions that are either in, or similar to, field conditions (e.g. nutrient media that closely resembles soil environment), as opposed to the highly artificial Petri dishes used in the majority of existing studies (Choi et al. 2014; Brilli et al. 2019; Song et al. 2019).

To gain a comprehensive understanding of the role of BVCs, it is necessary to study mutant strains that do not produce such compounds. Adverse effects from chemical treatments and difficulties in establishing optimal treatment concentrations restrict the use of chemicals for growth promotion and resistance induction in plants. Therefore, future research programs should focus on measuring VOC levels produced by rhizobacteria in soil ecosystems (Ryu et al. 2005b; Sharifi and Ryu 2018a). An understanding of the relevant signalling pathways and the extent to which they resemble plant responses to pathogens, and whether the physiological and molecular plant responses vary between bacterial species is crucial to aid our understanding and will determine future technology transfer in this area (Velivelli et al. 2015). In addition, genetically altering rhizobacterial strains to

produce greater amounts of volatiles that promote plant growth or that induce systemic resistance may represent promising avenues of future research. However, the use of GM organisms is becoming increasingly controversial in terms of human health and the environment. Phytopathogenic fungi—which are responsible for the majority of economically important crop diseases—are inhibited by BVCs, indicating that BVCs have the potential for use in agriculture as biological control agents. However, research on the effects of bacterial volatiles is still in the early stages (Sharifi and Ryu 2016).

In particular, it has yet to be determined whether the effects of volatiles are limited to specific plant tissues or if they affect plant development in general. Also, an understanding (positive or negative) of the impact of bacterial volatiles on plant metabolism is essential. Furthermore, it will be necessary to address whether effects observed in the laboratory transfer to the field (Velivelli et al. 2015). An alternative strategy that may prove beneficial for agriculture is the application of bio-active but affordable compounds, such as 2,3-butanediol, to aerial plant components to stimulate growth and ISR. Additional limitations on the use of rhizobacteria volatiles such as 2,3-butanediol and acetoin include the fact that they evaporate very quickly following application in the open field, however solutions are being developed to tackle this problem such as the use of microcapsules to slowly release

BVCs to the environment (Song and Ryu 2013; Sharifi and Ryu 2018b). Nevertheless, significant positive effects for BVCs have been comprehensively analysed under growth-chamber, greenhouse and open-field conditions (Velivelli et al. 2015; Choi et al. 2014). For example, pathogen growth in *A. thaliana* by *Pseudomonas syringae* was found to be considerably suppressed by the direct application of acetoin to roots under growth-chamber conditions (Rudrappa et al. 2010). Under greenhouse conditions, the soil-drench application of volatile metabolites, such as DMDS (released by *B. cereus* C1L), inhibited the activities of *Botrytis cinerea* and *Cochliobolus heterostrophus* against tobacco and corn plants, respectively (Huang et al. 2012). Pre-treatment of cucumber plants with 3-pentanol and 2-butanone showed protective benefits against the biotrophic bacterial pathogen *P. syringae* in open-field trials. (Song and Ryu 2013; Velivelli et al. 2015). Notwithstanding the challenges of transitioning from laboratory to field with respect to the application of mVOCs (Bailly and Weisskopf 2017) the studies conducted to date should lead to new discoveries regarding the use of VOCs to control microbial pathogens under open-field conditions and can be expected to act as big players in any second green revolution.

References:

Ahmed E, Holmström SJM (2014) Siderophores in environmental research: roles and applications. Microb Biotech 7:196–208

Almenar E, Del Valle V, Catala R, Gavara R (2007) Active package for wild strawberry fruit (*Fragaria vesca* L.). J Agric Food Chem 55:2240–2245

Arrebola E, Sivakumar D, Korsten L (2010) Effect of volatile compounds produced by Bacillus strains on postharvest decay in citrus. Biol Cont 53:122–128

Athukorala SNP, Fernando WGD, Rashid KY, De Kievit T (2010) The role of volatile and non-volatile antibiotics produced by *Pseudomonas chlororaphis* strain PA23 in its root colonization and control of *Sclerotinia sclerotiorum*. Biocont Sci Technol 20:875–890

Bailly A, Weisskopf L (2012) The modulating effect of bacterial volatiles on plant growth: current knowledge and future challenges. Plant Signal Behav 7:79–85

Bailly A, Weisskopf L (2017) Mining the volatilomes of plant-associated microbiota for new biocontrol solutions. Front Microbiol 8:1638

Besset-Manzoni Y, Rieusset L, Joly P, Comte G, Prigent-Combaret C (2018) Exploiting rhizosphere microbial cooperation for developing sustainable agriculture strategies. Environ Sci Pollut Res 25:29953

Biondi E, Blasioli S, Galeone A, Spinelli F, Cellini A, Luchese C, Braschi I (2014) Detection of potato brown rot and ring rot by electronic nose: From laboratory to real scale. Talanta 129:422–430

Blom D, Fabbri C, Connor EC, Schiestl FP, Klauser DR, Boller T, Eberl L, Weisskopf L (2011a) Production of plant growth modulating volatiles is widespread among rhizosphere bacteria

and strongly depends on culture conditions. Environ Microbiol 13:3047–3058

Blom D, Fabbri C, Eberl L, Weisskopf L (2011b) Volatile-mediated killing of *Arabidopsis thaliana* by bacteria is mainly due to hydrogen cyanide. Appl Environ Microbiol 77:1000– 1008

Blumer C, Haas D (2000) Iron regulation of the hcnABC genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. Microbiol 146:2417–2424

Brilli F, Loreto F, Baccelli I (2019) Exploiting Plant Volatile Organic Compounds (VOCs) in Agriculture to Improve Sustainable Defense Strategies and Productivity of Crops. Front Plant Sci 10:264

Carmen Orozco-Mosqueda M, Macías-Rodríguez L, Santoyo G, Farías-Rodríguez R, Valencia-Cantero E (2013) *Medicago truncatula* increases its iron-uptake mechanisms in response to volatile organic compounds produced by *Sinorhizobium meliloti*. Folia Microbiol 58:579–585

Cernava T, Müller H, Aschenbrenner IA, Grube M, Berg G (2015) Analysing the antagonistic potential of the lichen microbiome against pathogens by bridging 112on-pathogen with culture studies. Front Microbiol 6:620

Chen H, Xiao X, Wang J, Wu L, Zheng Z, Yu Z (2008) Antagonistic effects of volatiles generated by *Bacillus subtilis* on spore germination and hyphal growth of the plant pathogen, *Botrytis cinerea*. Biotechnol Lett 30:919–923

Cho G, Kim J, Park CG, Nislow C, Weller DM, Kwak Y-S (2017) Caryolan-1-ol, an antifungal volatile produced by *Streptomyces* spp., inhibits the endomembrane system of fungi. Open Biol 7:170075

Cho SM, Kang BR, Han SH, Anderson AJ, Park J-Y, Lee Y-H, Cho BH, Yang K-Y, Ryu C-M, Kim YC (2008) 2R,3R-Butanediol, a bacterial volatile produced by *Pseudomonas chlororaphis* O6, is involved in induction of systemic tolerance to

drought in *Arabidopsis thaliana*. Mol Plant Microbe Interact 21:1067–1075

Choi HK, Song GC, Yi HS, Ryu C-M (2014) Field evaluation of the bacterial volatile derivative 3-pentanol in priming for induced resistance in pepper. J Chem Ecol 40:882–892

Chuankun X, Minghe M, Leming Z, Keqin Z (2004) Soil volatile fungistasis and volatile fungistatic compounds. Soil Biol Biochem 36:1997–2004

Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959

Coosemans J (2005) Dimethyl disulphide (DMDS): a potential novel nematicide and soil disinfectant. Acta Hortic (ISHS) 698:57–64

Dandurishvili N, Toklikishvili N, Ovadis M, Eliashvili P, Giorgobiani N, Keshelava R, Tediashvili M, Vainstein A, Khmel I, Szegedi E, Chernin L (2011) Broad-range antagonistic rhizobacteria *Pseudomonas fluorescens* and *Serratia plymuthica* suppress *Agrobacterium* crown gall tumours on tomato plants. J Appl Microbiol 110:341–352

Dimkić I, Stanković S, Nišavić M, Petković M, Ristivojević P, Fira D, Berić T (2017) The profile and antimicrobial activity of *Bacillus* lipopeptide extracts of five potential biocontrol strains. Front Microbiol 8:925

Effmert U, Kalderás J, Warnke R, Piechulla B (2012) Volatile mediated interactions between bacteria and fungi in the soil. J Chem Ecol 38:665–703

Farag MA, Ryu C-M, Sumner LW, Paré PW (2006) GC–MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry 67:2262–2268

Farag MA, Song GC, Park Y-S, Audrain B, Lee S, Ghigo JM, Kloepper JW, Ryu C-M (2017) Biological and chemical strategies for exploring inter- and intra-kingdom communication mediated via bacterial volatile signals. Nat Protoc 12:1359–1377

Fernando WGD, Ramarathnam R, Krishnamoorthy AS, Savchuk SC (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biol Biochem 37:955–964

Fiddaman PJ, Rossall S (1993) The production of antifungal volatiles by *Bacillus subtilis*. J Appl Bacteriol 74:119–126

Fiddaman PJ, Rossall S (1994) Effect of substrate on the production of antifungal volatiles from *Bacillus subtilis*. J Appl Bacteriol 76:395–405

Fincheira P, Quiroz A (2018) Microbial volatiles as plant growth inducers. Microbiol Res 208:63– 75

Fritsch J (2005) Dimethyl 114on-pathog as a new chemical potential alternative to methyl bromide in soil disinfestation in France. Acta Hortic (ISHS) 698:71–76

Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 69:30–39

Groenhagen U, Baumgartner R, Bailly A, Gardiner A, Eberl L, Schulz S, Weisskopf L (2013) Production of bioactive volatiles by different *Burkholderia ambifaria* Strains. J Chem Ecol 39:892–906

Grosch R, Faltin F, Lottmann J, Kofoet A, Berg G (2005) Effectiveness of 3 antagonistic bacterial isolates to control *Rhizoctonia solani* Kühn on lettuce and potato. Can J Microbiol 51:345–353

Gu Y-Q, Mo M-H, Zhou J-P, Zou C-S, Zhang K-Q (2007) Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. Soil Biol Biochem 39:2567–2575

Gutiérrez-Luna F, López-Bucio J, Altamirano-Hernández J, Valencia-Cantero E, Cruz H, Macías-Rodríguez L (2010) Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis 51:75–83

Hérnandez-Calderón E, Aviles-Garcia ME, Castulo-Rubio DY, Macías-Rodríguez L, Ramírez VM, Santoyo G, López-Bucio J, Valencia-Cantero E (2018) Volatile compounds from beneficial or pathogenic bacteria differentially regulate root exudation, transcription of iron transporters, and defense 115on-pathog pathways in *Sorghum bicolor*. Plant Mol Biol 96:291–304

Howell CR, Beier RC, Stipanovic RD (1988) Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. Phytopathology 78:1075–1078

Huang C-J, Tsay J-F, Chang S-Y, Yang H-P, Wu W-S, Chen C-Y (2012) Dimethyl 115on-pathog is an induced systemic resistance elicitor produced by *Bacillus cereus* C1L. Pest Manag Sci 68:1306–1310

Huang Y, Xu C, Ma L, Zhang K, Duan C, Mo M (2010) Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against *Meloidogyne incognita*. Euro J Plant Pathol 126:417–422

Jeong H, Choi S-K, Ryu C-M, Park S-H (2019) Chronicle of a soil bacterium: *paenibacillus polymyxa* E681 as a tiny guardian of plant and human health. Front Microbiol 10:467

Kai M, Crespo E, Cristescu SM, Harren FJ, Francke W, Piechulla B (2010) *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. Appl Microbiol Biotechnol 88:965–976

Kai M, Effmert U, Berg G, Piechulla B (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Arch Microbiol 187:351–360

Kai M, Haustein M, Molina F, Petri A, Scholz B, Piechulla B (2009) Bacterial volatiles and their action potential. Appl Microbiol Biotechnol 81:1001–1012

Kai M, Piechulla B (2009) Plant growth promotion due to rhizobacterial volatiles – An effect of CO_2? FEBS Lett 583:3473–3477

Kai M, Piechulla B (2010) Impact of volatiles of the rhizobacteria *Serratia odorifera* on the moss *Physcomitrella patens*. Plant Signal Behav 5:444–446

Kai M, Effmert U, Piechulla B (2016) Bacterial-plant-interactions: approaches to unravel the biological function of bacterial volatiles in the rhizosphere. Front Microbiol 7

Kanchiswamy CN, Malnoy M, Maffei M (2015) Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Front Plant Sci 6:151

Kim J-S, Lee J, Seo S-J, Lee C, Woo SY, Kim S-H (2015) Gene expression profile affected by volatiles of new plant growth promoting rhizobacteria, *Bacillus subtilis* strain JS, in tobacco. Genes Genom 37:387–397

Kloepper JW, Rodríguez-Kábana R, Zehnder AW, Murphy JF, Sikora E, Fernández C (1999) Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. Australas Plant Pathol 28:21–26

Kloepper JW and Schroth MN (1978) Plant growth promoting rhizobacteria on radishes. In: Proceedings of the 4th international conference on plant pathogenic bacteria, Angers, France, vol 2, pp 879–882

Kurze S, Bahl H, Dahl R, Berg G (2001) Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Dis 85:529–534

Kwon Y, Ryu C-M, Lee S, Park H, Han K, Lee J, Lee K, Chung W, Jeong M-J, Kim H, Bae D-W (2010) Proteome analysis of

Arabidopsis seedlings exposed to bacterial volatiles. Planta 232:1355–1370

Leach J, Triplett LR, Argueso CT, Trivedi P (2017) Communication in the phytobiome. Cell 169 (4):587–596

Lee B, Farag MA, Park HB, Kloepper JW, Lee SH, Ryu C-M (2012) Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa*. PloS One 7:e48744

Liu H, Brettell LE (2019) Plant defense by VOC-induced microbial priming. Trends Plant Sci 24:187–189

Liu W, Zhao L, Wang C, Mu W, Liu F (2009) Bioactive evaluation and application of antifungal volatiles generated by five soil bacteria. Acta Phytophyl Sin 36:97–105

López-Bucio J, Campos-Cuevas JC, Hernández-Calderón E, Velásquez-Becerra C, Farías-Rodríguez R, Macías-Rodríguez LI, Valencia-Cantero E (2007) *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-independent signalling mechanism in *Arabidopsis thaliana*. Mol Plant Microbe Interact 20:207–217

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Ann Rev Microbiol 63:541–556

Marquez-Villavicencio MDP, Weber B, Witherell RA, Willis DK, Charkowski AO (2011) The 3-Hydroxy-2-Butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. PloS One 6:e22974

Müller H, Westendorf C, Leitner E, Chernin L, Riedel K, Schmidt S, Eberl L, Berg G (2009) Quorum-sensing effects in the antagonistic rhizosphere bacterium *Serratia plymuthica* HRO-C48. FEMS Microbiol Ecol 67:468–478

Nawrath T, Mgode GF, Weetjens B, Kaufmann SH, Schulz S (2012) The volatiles of pathogenic and non-pathogenic

mycobacteria and related bacteria. Beilstein J Org Chem 8:290–299

Nicholson WL (2008) The *Bacillus subtilis* ydjL (*bdhA*) gene encodes acetoin reductase/ 2,3-Butanediol dehydrogenase. Appl Environ Microbiol 74:6832–6838

Oteino N, Lally RD, Kiwanuka S, Lloyd A, Ryan D, Germaine KJ, Dowling DN (2015) Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. Front Microbiol 6:745

Park Y-S, Dutta S, Ann M, Raajmakers JM, Park K (2015) Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. Biochem Biophys Res Commun 461:361–365

Park HB, Lee B, Kloepper JW, Ryu CM (2013) One shot-two pathogens blocked: exposure of *Arabidopsis* to hexadecane, a long chain volatile organic compound, confers induced resistance against both *Pectobacterium carotovorum* and *Pseudomonas syringae*. Plant Signal Behav 8: e24619

Rath M, Mitchell TR, Gold SE (2018) Volatiles produced by *Bacillus mojavensis* RRC101 act as plant growth modulators and are strongly culture-dependent. Microbiol Res 208:76–84

Rijavec T, Lapanje A (2016) Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. Front Microbiol 7:1785

Romera FJ, García MJ, Lucena C, Martínez-Medina A, Aparicio MA, Ramos J, Alcántara E, Angulo M, Pérez-Vicente R (2019) Induced systemic resistance (ISR) and fe deficiency responses in dicot plants. Front Plant Sci 10:287

Rosier A, Medeiros FHV, Bais HP (2018) Defining plant growth promoting rhizobacteria molecular and biochemical networks in beneficial plant-microbe interactions. Plant Soil 428:35

Rudrappa T, Biedrzycki ML, Kunjeti SG, Donofrio NM, Czymmek KJ, Paul WP, Bais HP (2010) The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana*. Commun Integr Biol 3:130–138

Ryu C-M, Farag MA, Hu C-H, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017–1026

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017–1026 (Published correction appears in Plant Physiol. 2005 Apr;137:1486)

Ryu C-M, Farag MA, Hu C-H, Reddy MS, Wei H-X, Paré PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci 100:4927–4932

Ryu C-M, Hu C-H, Locy R, Kloepper J (2005a) Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. Plant Soil 268:285–292

Ryu C-M, Farag MA, Paré PW, Kloepper JW (2005b) Invisible signals from the underground: bacterial volatiles elicit plant growth promotion and induce systemic resistance. Plant Pathol J 21:7–12

Sang MK, Kim JD, Kim BS, Kim KD (2011) Root treatment with rhizobacteria antagonistic to *Phytophthora* blight affects anthracnose occurrence, ripening, and yield of pepper fruit in the plastic house and field. Phytopathology 101:666–678

Santoro MV, Zygadlo J, Giordano W, Banchio E (2011) Volatile organic compounds from rhizobacteria increase biosynthesis of essential oils and growth parameters in peppermint (*Mentha piperita*). Plant Physiol Biochem 49:1177–1182

Schulz-Bohm K (2017) Microbial volatiles: small molecules with an important role in intra- and inter-kingdom interactions. Front Microbiol 8:2484

Shao J, Xu Z, Zhang N, Shen Q, Zhang R (2015) Contribution of indole-3-acetic acid in the plant growth promotion by the rhizospheric strain *Bacillus amyloliquefaciens* SQR9. Biol Fertil Soils 51:321

Sharifi R, Ryu CM (2016) Making healthier or killing enemies? Bacterial volatile-elicited plant immunity plays major role upon protection of *Arabidopsis* than the direct pathogen inhibition. Commun Integr Biol 9:e1197445

Sharifi R, Ryu C-M (2018a) Revisiting bacterial volatile-mediated plant growth promotion: lessons from the past and objectives for the future. Ann Bot 122:349–358

Sharifi R, Ryu C-M (2018b) Biogenic volatile compounds for plant disease diagnosis and health improvement. Plant Pathol J 34:459–469

Singh S (2014) A review on possible elicitor molecules of cyanobacteria: their role in improving plant growth and providing tolerance against biotic or abiotic stress. J Appl Microbiol 117:1221–1244

Song G, Ryu C-M (2013) Two volatile organic compounds trigger plant self-defense against a bacterial pathogen and a sucking insect in cucumber under open field conditions. Int J Mol Sci 14:9803–9819

Song GC, Ryu CM (2018) Evidence for Volatile Memory in Plants: Boosting Defence Priming through the Recurrent Application of Plant Volatiles. Mol. Cells 41(8):724

Song GC, Riu M, Ryu C-M (2019) Beyond the two compartments Petri-dish: optimising growth promotion and induced resistance in cucumber exposed to gaseous bacterial volatiles in a miniature greenhouse system. Plant Methods 15:9

Spinelli F, Cellini A, Vanneste J, Rodriguez-Estrada M, Costa G, Savioli S, Harren FM, Cristescu S (2012) Emission of volatile compounds by *Erwinia amylovora*: biological activity in vitro and possible exploitation for bacterial identification. Trees 26:141–152

Spinelli F, Costa G, Rondelli E, Busi S, Vanneste JL, Rodriguez EMT, Savioli S, Harren FJM, Crespo E, Cristescu SM (2011) Emission of volatiles during the pathogenic interaction between *Erwinia amylovora* and *Malus domestica*. Acta Hortic (ISHS) 896:55–63

Spinelli F, Noferini M, Vanneste JL, Costa G (2010) Potential of the electronic-nose for the diagnosis of bacterial and fungal diseases in fruit trees. EPPO Bull 40:59–67

Tahir JAS, Gu Q, Wu J, Niu Y, Huo R, Gao X (2017a) *Bacillus* volatiles adversely affect the physiology and ultra-structure of *Ralstonia solanacearum* and induce systemic resistance in tobacco against bacterial wilt. Sci Rep 7:40481

Tahir JAS, Gu Q, Wu J, Raza W, Hanif A, Wu L, Colman MV, Gao X (2017b) Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. Front Microbiol 8:171

Tahir JAS, Gu Q, Wu H, Raza W, Safdar A, Huang Z, Rajer FU, Gao X (2017c) Effect of volatile compounds produced by *Ralstonia solanacearum* on plant growth promoting and systemic resistance inducing potential of *Bacillus* volatiles. BMC Plant Biol 17:133

Tenorio-Salgado S, Tinoco R, Vazquez-Duhalt R, Caballero-Mellado J, Perez-Rueda E (2013) Identification of volatile compounds produced by the bacterium *Burkholderia tropica* that inhibit the growth of fungal pathogens. Bioengineered 4:236–243

Ting A, Mah S, Tee C (2011) Detection of potential volatile inhibitory compounds produced by endobacteria with biocontrol properties towards *Fusarium oxysporum* f. sp. *cubense* race 4. World J Microbiol Biotechnol 27:229–235

Van Der Kooij LAW, Kok LJD, Stulen I (1999) Biomass production and carbohydrate content of *Arabidopsis thaliana* at atmospheric CO_2 concentrations from 390 to 1680 ll l-1. Plant Biol 1:482–486

Velázquez-Becerra C, Macías-Rodríguez L, López-Buci J, Altamirano-Hernández J, Flores-Cortez I, Valencia-Cantero E (2011) A volatile organic compound analysis from Arthrobacter agilis identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. Plant Soil 339:329–340

Velázquez-Becerra C, Macías-Rodríguez L, López-Bucio J, Flores-Cortez I, Santoyo G, Hernández-Soberano C, Valencia-Cantero E (2013) The rhizobacterium *Arthrobacter agilis* produces dimethylhexadecylamine, a compound that inhibits growth of phytopathogenic fungi in vitro. Protoplasma 250:1251–1262

Velivelli SL, Sessitch A, Doyle Prestwich B (2014) The role of microbial inoculants in integrated crop management systems. Potato Res 57:291–309

Velivelli SL, Kromann P, Lojan P, Rojas M, Franco J, Suarez JP, Doyle Prestwich B (2015) Identification of mVOCs from Andean rhizobacteria and field evaluation of bacterial and mycorrhizal inoculants on growth of potato in its center of origin. Microb Ecol 69:652–667

Vespermann A, Kai M, Piechulla B (2007) Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Appl Environ Microbiol 73:5639–5641

Voisard C, Keel C, Haas D, Defago G (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J 8:351–358

Wan M, Li G, Zhang J, Jiang D, Huang H-C (2008) Effect of volatile substances of *Streptomyces platensis* F-1 on control of plant fungal diseases. Biol Control 46:552–559

Ward JK, Strain BR (1999) Elevated CO_2 studies: past, present and future. Tree Physiol 19:211– 220

Weise T, Kai M, Gummesson A, Troeger A, Von Reuss S, Piepenborn S, Kosterka F, Sklorz M, Zimmermann R, Francke W, Piechulla B (2012) Volatile organic compounds produced by the

phytopathogenic bacterium *Xanthomonas campestris pv. Vesicatoria* 85-10. Beilstein J Org Chem 8:579–596

Weise T, Kai M, Piechulla B (2013) Bacterial ammonia causes significant plant growth inhibition. PloS One 8:e63538

Weisskopf L, Ryu CM, Raaijmakers JM, Garbeva P (2016) Smelly fumes-volatile-mediated communication between bacteria and other organisms. Front. Microbiol. 7

Xie X, Zhang H, Pare PW (2009) Sustained growth promotion in *Arabidopsis* with long-term exposure to the beneficial soil bacterium *Bacillus subtilis* (GB03). Plant Signal Behav 4:948–953

Yang L-L, Huang Y, Liu J, Ma L, Mo M-H, Li W-J, Yang F-X (2012) *Lysinibacillus mangiferahumi* sp. nov., a new bacterium producing nematicidal volatiles. Antonie Van Leeuwenhoek 102:53–59

Yaoyao E, Yuan J, Yang F, Wang L, Ma J, Li J, Pu X, Raza W, Hwang Q, Shen Q (2017) PGPR strain *Paenibacillus polymyxa* SQR-21 potentially benefts watermelon growth by re-shaping root protein expression. AMB Expr 7:104

Yu SM, Lee Y (2013) Plant growth promoting rhizobacterium *Proteus vulgaris* JBLS202 stimulates the seedling growth of Chinese cabbage through indole emission. Plant Soil 370:485–495

Yuan J, Raza W, Shen Q, Huang Q (2012) Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. Appl Environ Microbiol 78:5942–5944

Yuan J, Zhao M, Li R, Huang Q, Raza W, Rensing C, Shen Q (2017) Microbial volatile compounds alter the soil microbial community. Environ Sci Pollut Res 24:22485–22493

Zhang H, Kim MS, Sun Y, Dowd SE, Shi H, Paré PW (2008a) Soil bacteria confer plant salt tolerance by tissue-specific

regulation of the sodium transporter HKT1. Mol Plant Microbe Interact 21:737–744

Zhang H, Kim MS, Krishnamachari V, Payton P, Sun Y, Grimson M, Farag MA, Ryu CM, Allen R, Melo IS, Pare PW (2007) Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. Planta 226:839–851

Zhang H, Sun Y, Xie X, Kim MS, Dowd SE, Paré PW (2009) A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. Plant J 58:568–577

Zhang H, Xie X, Kim MS, Kornyeyev DA, Holaday S, Pare PW (2008b) Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. Plant J 56:264–273

Zhang X, Li B, Wang Y, Guo Q, Lu X, Li S, Ma P (2013) Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. Appl Microbiol Biotechnol 97:9525–9534

Zhao L-J, Yang X-N, Li X-Y, Mu W, Liu F (2011) Antifungal, insecticidal and herbicidal properties of volatile components from *Paenibacillus polymyxa* strain BMP-11. Agric Sci China 10:728–736

Zou C, Li Z, Yu D (2010) *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran. J Microbiol 48:460–466

Chapter 3

Help from Below: Screening of Irish Potato Rhizosphere Bacteria for Plant Growth-Promoting Activities from Organic and Conventional Cropping Systems

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Abstract:

The need for new solutions to current methods of food production across the world is a significant problem facing the agricultural industry. In an effort to address this problem an isolation campaign was carried out across the Southern coast of Ireland to identify plant growth-promoting rhizobacteria (PGPR) associated with potato (*Solanum tuberosum*) from both organic and conventional cropping systems. By their nature, PGPR may present a viable alternative to synthetic plant protection products (PPP). Direct and indirect PGP activities were investigated, isolates were subjected to a number of biochemical screening tests for the ability to produce bacterial volatile organic compounds (BVCs), hydrogen cyanide (HCN), ammonia (NH_3), siderophores and solubilisation of tricalcium phosphate. The 21 selected bacterial isolates were identified using 16S rRNA gene

analysis. Here we report on the plant growth-promoting (PGP) activities of 21 Irish potato soil isolates from diverse genera such as *Bacillus*, *Pseudomonas*, *Serratia* and *Achromobacter* obtained from four potato cultivars. We found considerable overlap in the isolates obtained from both conventional and organic sites, with identical members of *Bacillus*, *Achromobacter* and *Pseudomonas* identified from both cropping regimes. One isolate identified from both cropping regimes, *Pseudomonas helmanticensis*, was capable of all five biochemical PGP activities. To the best of our knowledge, this is the first analysis of the potential of Irish potato soils to act as a 'biobank' for PGPR candidates. This study provides a grounding for further analyses on the microbiome of Irish potato soils and a snapshot of the PGPR community structure within the wider soil microbiome.

Introduction:

Over the past four decades there has been an intense amount of research focused on the interaction of PGPR with their respective plant hosts (Kloepper and Schroth, 1978; Wei et al. 1996; Lucy et al. 2004; Ryu et al. 2004). The vast range of studies conducted thus far has helped to elucidate some of the key molecular mechanisms used by PGPR to aid the growth and overall health status of their associated hosts, these mechanisms have been optimised over hundreds of millions of years of co-evolution since the establishment of plants on land (Warshan et al.

2018). Today's world is placed on the precipice of a possible climate catastrophe within the next century, largely due to carbon-intensive and environmentally-destructive conventional agricultural practices (Smith et al. 2014). An ever-burgeoning global population requires a constant supply of food, while at the same time in markets such as that of the European Union, there is a push to limit both the amount and scope of synthetic biocides and fertilisers required to maintain this food supply. Therefore, PGPR and/or their respective metabolites may provide an opportunity to employ a more sustainable and environmentally-friendly approach to food production (Velivelli et al. 2014; Vejan et al. 2016).

Potato (*Solanum tuberosum*) is the world's most widely-grown tuber crop along with cassava (*Manihot esculenta*) which together cover 4% of the world's total harvested area (Leff et al. 2014; Devaux et al. 2021). Crucially, due to potato's high biomass yield per hectare compared to other crops, it has the potential to increase the micronutrient status of consumers in socio-economically deprived countries via PGPR-mediated biofortification for micronutrients such as iron and zinc (Daly et al. 2017). Although the centre of origin for potato lies in the Andean regions of South America (Ghyselinck et al. 2013), it is often infamously associated with the Irish potato famine which lasted from 1845-1849 and was responsible for over one million deaths and a further one million people emigrating; only in recent years has the country's population begun to recover towards pre-potato famine levels (Fotheringham et al. 2013). Today, fungal

phytopathogens are still a major concern and the arms race between fungus and farmer is as intense as ever and the phytopathogenic fungus *Rhizoctonia solani* which is responsible for the diseases; black scurf and stem canker, is one which represents a significant challenge to the worlds agricultural potato yield (Kanetis et al. 2016). Many phytopathogens, especially fungal phytopathogens, thrive in conditions which are humid and relatively warm (Talley et al. 2002), conditions which are becoming more prevalent in North-Western Europe and will likely continue to do so due to climate change. Therefore, a measured response to this threat is required, a response which has both the potential to meet ambitious EU regulations on PPP and continue to maintain current food production levels in the face of classical and emerging crop disease (Savary et al. 2019). The aim of this study was to investigate Irish potato soils from both organic and conventional cropping systems for the presence of potential PGPR and to also give a preliminary insight into the bacterial community composition between organic and conventional cropping regimes in Ireland. The functional diversity of bacteria in Irish potato ridge and bulk soils has been reported previously (Lettice and Jones, 2017) but currently there is little to no information regarding the ability of rhizobacteria isolated from Irish potato soils to act as potential biocontrol agents. To this end, three sites were selected across the Southern coast of Ireland from which to obtain soil samples; two organic sites and one conventional site. Various PGP activities were selected for; in the first instance, isolates were screened for their ability to inhibit the growth of *R. solani*

in an in vitro BVC-mediated antagonistic assay. Following this, successful candidates were tested for four other major PGP activities, the ability to produce hydrogen cyanide (HCN) to potentially inhibit fungal phytopathogens (Flaishman et al. 1996), ammonia (NH_3) which can aid in root knot nematode biocontrol activity for example (Oka et al. 1993), siderophores which can potentially capture and sequester iron from the rhizosphere to biofortify crops and deny access of iron to phytopathogens for growth (Radzki et al. 2013; Sayeed et al. 2013) and to solubilise tricalcium phosphate for plant uptake of phosphates required for plant growth (Chabot et al. 1998; Billah et al. 2019). All selected bacterial isolates were identified through 16S rRNA gene sequence analysis.

Materials and Methods:

Isolation of Rhizosphere Bacteria:

Rhizospheric soil samples were collected from both organic and conventional *Solanum tuberosum* crops during peak summer growth season across the Southern coast of Ireland (June 2015). The three collection sites and respective cultivars are as follows; 1) Liss Ard Estate, Skibbereen, Cork (Organic) cultivars; 'Maris Bard' and 'Colleen'. 2) Barryroe, Clonakilty, Cork (Conventional) cultivars; 'Premiere' and 'British Queen'. 3) Ballymaloe Cookery School, Shanagarry, Cork, cultivar; 'Colleen'. Six plants of each cultivar were

collected from both the leading edge and centre of each respective crop. Plants were immediately sealed in sterile autoclave bags and kept at 4 °C overnight in the dark. The following day, using an adapted methodology from Ghyselinck et al. (2013), 5 g of soil adhering directly to the plant root was collected and pooled per cultivar (total 30 g of soil per 6 plants). Following this 1g of pooled soil was placed in 5 ml phosphate buffered saline, three sterile ~5 mm glass beads were added and the solution was vortexed for 1 minute. Serial dilutions were prepared from these solutions ranging from 10^{-1} to 10^{-6} and 100 µl of each respective dilution was spread-plated on ¼ strength tryptic soy agar (TSA) (Sigma Aldrich, Ireland) supplemented with 0.05% cycloheximide w/v (Sigma Aldrich, Ireland) to inhibit fungal growth. Petri dishes were placed in incubators at both 28 ± 2 °C and room temperature. Bacterial growth was observed for two weeks and distinct bacterial colonies were picked off using an inoculation loop under aseptic conditions and streaked to purity on full-strength TSA as they appeared visible to the naked eye.

Bacterial volatile-mediated growth-inhibition of Rhizoctonia solani:

Cultures of *R. solani* EC-1 supplied by the school of Biological, Earth and Environmental Sciences, UCC, Ireland, were prepared by placing a single ~8 mm plug of a two-week-old fungal culture in the centre of a Petri plate containing potato dextrose agar (PDA) (Sigma Aldrich, Ireland) and allowed to grow for two weeks at 25 ± 2 °C. Following

this, two-compartment petri dishes (I-plates) containing both TSA and PDA in respective compartments were prepared. A single ~8 mm plug of *R. solani* was placed at the leading edge of the compartment containing PDA. In the opposite compartment, a single bacterial colony from an overnight culture grown at 28 ± 2 °C on TSA was streaked across the media. The plate was then sealed with Parafilm and carefully placed upside down in an incubator at 25 ± 2 °C. At the same time control plates were prepared which lacked any bacterial isolates. When the fungal growth in the control plates reached the dividing wall of the I-plate, measurements of fungal growth in test plates were conducted. The calculation of inhibition efficiency was determined according to Ghyselinck et al. (2013). Tests were performed five times per isolate.

Hydrogen Cyanide (HCN) production:

Bacterial isolates were grown on nutrient agar (Sigma Aldrich, Ireland) supplemented with 4.4 g glycine (Sigma Aldrich, Ireland) per litre. A 5 cm strip of picric acid paper was placed in the lid of the Petri dish and the dish was then sealed with Parafilm. Plates were then placed upside down in an incubator at 28 ± 2 °C for four days. Colour change of the picric acid paper from yellow to orange-red indicated HCN production. Tests were performed twice per isolate.

Ammonia (NH_3) production:

A single colony of bacteria grown on TSA overnight at 28 ± 2°C was added to 10 ml of Peptone water (Sigma Aldrich, Ireland) and incubated at 28 ± 2 °C and 170 rpm for 72 h. Following this, 0.5 ml of Nessler's reagent (Sigma Aldrich, Ireland) was added (Cappuccino and Sherman, 1992). Development of a dark orange/brown colour indicated ammonia production. Tests were performed twice per isolate.

Phosphate solubilisation:

Phosphate solubilisation activity was assessed by growing bacterial cultures on NBRIP phosphate growth media (Nautiyal, 1999) containing, per litre, glucose (10 g); $Ca_3(PO_4)_2$ (5 g); $MgCl_2 \cdot 6H_2O$ (5 g); $MgSO_4 \cdot 7H_2O$ (0.25 g); KCl (0.2 g) and $(NH_4)_2SO_4$ (0.1 g) (Sigma Aldrich, Ireland). Briefly, single bacterial colonies from overnight-grown cultures at 28 ± 2 °C on TSA were stabbed directly into the growth media using a sterile 200 µl pipette tip. After a period of 25 days radial zones of clearance were measured around the original stab point to determine the rate of phosphate solubilisation. There were four replicates and the experiment was repeated once.

Siderophore production:

Siderophore production was assessed using an adapted O-CAS methodology (Pérez-Miranda et al. 2007). Bacterial isolates were

grown on their optimal media, in this instance TSA. A single colony of bacteria was placed in 10 ml of tryptic soy broth (TSB) (Sigma Aldrich, Ireland) in an incubator overnight at 28 ± 2 °C and 170 rpm. The following day 100 µl of this culture was spot-plated directly onto the centre of a Petri dish containing TSA and placed in an incubator at 28 °C ± 2 °C. Three days later, a layer of O-CAS media containing, per litre, Chrome azurol S (CAS) (60.5 mg); hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg); Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) (30.24 g) and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 ml and agarose (0.9%, w/v) (Sigma Aldrich, Ireland) was poured over the bacterial colony. Development of a red/orange halo around the colony after 24 h indicated a positive result for siderophore production. Prior to preparation of O-CAS media all glassware was rinsed with 6 M HCL in order to deferrate all surfaces. The test was performed twice per isolate.

Bacterial DNA extraction:

Briefly, a single colony of an overnight grown culture was inoculated under aseptic conditions into 5 ml liquid LB media in a 20 ml McCartney vial and allowed to grow overnight at 28 ± 2 °C and 170 rpm, a negative control lacking a bacterial colony was also prepared. The following day DNA was extracted from 2 ml of this culture according to the QIAGEN® DNeasy® Blood and Tissue Kit "Quick-Start Protocol".

Sequencing and Identification of Bacterial Isolates:

A fragment of the 16S ribosomal RNA gene was amplified in each sample by PCR using the primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Lane et al. 1985). PCR was performed in 20 µl volumes using 10 µl of 2X TopBio PP Combi PCR mastermix, 1µM each of forward and reverse primers and 1 µl of template DNA. Cycling conditions were as follows: An initial denaturation step of three minutes at 95 °C, followed by 35 cycles of 94 °C for 30s, 54 °C for 60s and 72 °C for 90s. A final extension step of 72 °C for five minutes was included to stabilise the reaction. PCR products were run on 1% agarose stained with SYBRsafe™ (Invitrogen®) gel stain. Once it was established that negative controls were clear of any contamination, products from the cultures were excised and purified for sequencing by centrifugation at high speed through glass wool for three minutes. Sequencing was performed from both ends of the product using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®). Sequencing reactions were purified using the EDTA-ethanol precipitation method described in the sequencing kit handbook and were run on an ABI3500XL DNA analyser. Sequencing output quality (raw traces) was analysed using Chromas software 2.6.6.(Technelysium Pty Ltd) and low quality sequence was trimmed and deleted. Resulting forward and reverse 16S rRNA sequences were assembled using Sequencher 5.4.6. (Gene Codes Corporation). Following this, non-DNA bases were removed and

consensus sequences were queried against the EzBioCloud 16S rRNA database for genus/species identification (Yoon et al. 2012). Any similarity scores above 98.6% were taken to indicate a genus/species match to the database. Sequences have been deposited in GenBank with accession numbers MT373391-MT373411.

Statistical analyses:

Tests for normality and statistical analyses were carried out using SPSS 25 (IBM). To analyse the statistical significance between isolates capable of phosphate solubilisation, the Kruskal-Wallis test was performed. One-way analysis of variance (ANOVA) was used to determine the effect of isolate on the growth-inhibition of *R. solani*.

Results:

Isolation and 16S rRNA Sequence Identification of Bacteria:

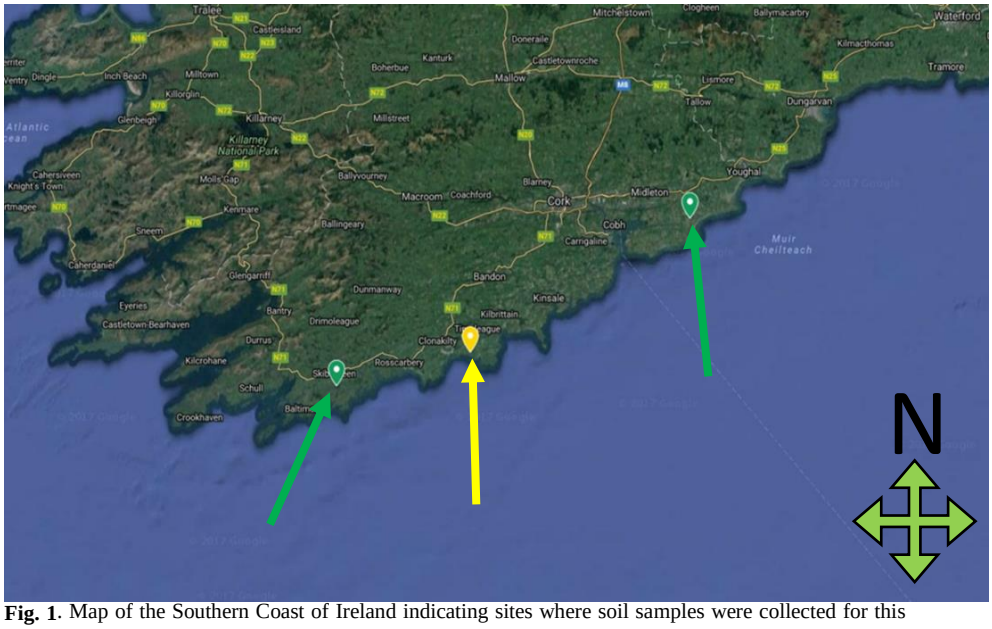


Fig. 1. Map of the Southern Coast of Ireland indicating sites where soil samples were collected for this study.Organic cropping sites are displayed with green arrows (Liss Ard to the left of the image; Ballymaloe Cookery School to the right). The conventional cropping site, located in Barryroe is displayed with a yellow arrow. Satellite image credit; Google maps.

There were 21 isolates recovered from the three test sites tested for PGP activities (Fig. 1), 11 from organic crop sites and 10 from the conventional crop site. From the organic sites, Liss Ard soil samples were collected from two cultivars named ‘Colleen’ and ‘Maris Bard’, three isolates were obtained from Colleen and three were obtained from Maris Bard. At Ballymaloe Cookery School five isolates were obtained from soil samples also associated with the Colleen cultivar. Soil samples obtained from the conventional site in Barryroe were associated with two cultivars named ‘Premiere’ and ‘British Queen’. There were seven isolates obtained from Premiere and three were obtained from British Queen. Soils from all sites are reported by the

Irish Soil Survey to be coarse loamy drift with siliceous stones and a pH ranging from 5.54-5.70 (http://gis.teagasc.ie/soils/map.php)

Table 1. 16S rRNA identification of 21 selected bacterial isolates.

Sample Site and Co-ordinates	Cultivar	Isolate Designation	Bacterial Identification	Similarity (%)	Reference
Barryroe (51°35'52.1"N 8°47'36.9"W)	Premiere	BRP1	*Achromobacter marplatensis*	99.56	Gomila et al. (2011); Shi et al. (2017)
	Premiere	BRP4	*Stenotrophomonas rhizophila*	99.23	Wolf et al. (2002); Schmidt et al. (2012); Alavi et al. (2013)
	Premiere	BRP10	*Stenotrophomonas rhizophila*	99.51	Wolf et al. (2002); Schmidt et al. (2012); Alavi et al. (2013)
	Premiere	BRP14	*Pseudomonas azotoformans*	99.72	Elo et al. (2000); Sang et al. (2014)
	Premiere	BRP18	*Achromobacter marplatensis*	99.27	Gomila et al. (2011); Shi et al. (2017)
	Premiere	BRP37	*Glutamicibacter (Arthrobacter) bergerei*	98.65	Aslam and Ali (2018)
	Premiere	BRP48	*Pseudomonas helmanticensis*	98.52	Ramírez-Bahena et al. (2014)
	British Queen	BRQ6	*Rhodococcus erythropolis*	99.63	Elo et al. (2000); Trivedi et al. (2007); Cirou et al. (2011); Barbey et al. (2013)
	British Queen	BRQ10	*Bacillus mycoides*	98.46	Yi et al. (2017); Huang et al. (2018)

	British Queen	BRQ16	*Pseudomonas fragi*	99.22	Martin et al. (2007); Selvakumar et al. (2009)
Liss Ard ^ (51°31'49.6"N 9°15'16.3"W)	Maris Bard	LAM2	*Pseudomonas helmanticensis*	99.71	Ramírez-Bahena et al. (2014)
	Maris Bard	LAM7	*Bacillus mycoides*	99.23	Yi et al. (2017); Huang et al. (2018)
	Maris Bard	LAM10	*Serratia myotis*	99.14	García-Fraile et al. (2015)
	Colleen	LAC1	*Ochrobactrum thiophenivorans*	100.00	Kämpfer et al. (2008)
	Colleen	LAC2	*Serratia fonticola*	99.58	Devi et al. (2013); Kwon Jung et al. (2017)
	Colleen	LAC6	*Micrococcus luteus*	98.71	Raza and Faisal (2013); Lafi et al. (2017)
Ballymaloe Cookery School ^ (51°51'30.8"N 8°01'53.4"W)	Colleen	BMC3	*Achromobacter marplatensis*	99.70	Gomila et al. (2011); Shi et al. (2017)
	Colleen	BMC4	*Pseudomonas sediminis*	99.21	Behera et al. (2018)
	Colleen	BMC6	*Lysinibacillus macroides*	98.95	Suvega and Arunkumar 2019
	Colleen	BMC10	*Bacillus toyonensis*	99.08	Lopes et al. (2017); Agamennone et al. (2019)
	Colleen	BMC11	*Bacillus toyonensis*	99.37	Lopes et al. (2017); Agamennone et al. (2019)

Results of 16S rRNA gene sequence analysis using the EzBioCloud online database. Similarity scores above 98.6% are taken to be a match to the EzBioCloud database. (^) denotes sites of organic cropping regimes.

16S rRNA analysis revealed a wide array of genera from the phyla Proteobacteria, Actinobacteria and Firmicutes across all potato crop sites tested and identified many bacteria commonly associated with PGP in previous studies (Table 1). At the Liss Ard site in the Colleen cultivar there were two members of Proteobacteria (*Ochrobactrum* and *Serratia*) and one member of Actinobacteria (*Micrococcus*). At the same site, in the Maris Bard cultivar there were two members of the Proteobacteria (*Pseudomonas* and *Serratia*) and one member of the Firmicutes (*Bacillus*). At the Barryroe site in the Premiere cultivar, seven members of the Proteobacteria belonging to *Achromobacter* and *Pseudomonas* and one member of the Actinobacteria *Glutamicibacter* (*Arthrobacter*) were present. In the British Queen cultivar located at the same site there were two members of the Proteobacteria, one member of the Actinobacteria and one member of the Firmicutes namely *Pseudomonas*, *Rhodococcus* and *Bacillus* respectively. The members of *Stenotrophomonas* were only observed in the Premiere cultivar from the conventional site. From all sites combined, members of *Pseudomonas* comprised ~24% of sequenced isolates, *Bacillus* comprised ~19%, *Achromobacter* comprised ~14%, *Stenotrophomonas* and *Serratia* comprised 9.52% respectively, *Ochrobactrum*, *Rhodococcus*, *Micrococcus*, *Glutamicibacter*, and *Lysinibacillus* each comprised 4.8% of sequenced isolates.

Biochemical PGP characterisation of Selected Bacterial Isolates:

To determine the PGP potential of selected isolates a suite of five biochemical activities consisting of both direct and indirect PGP mechanisms were screened for, in vitro, as reported in previous studies (Ahmed et al. 2008). A scoring system out of a total of five was devised to determine the potential effectiveness of isolates for field application to respective crops (Table 2). In some cases, PGP activities such as phosphate solubilisation did not appear to be consistent across isolates deemed to be identical from 16S rRNA analysis, for example, in BRP4 and BRP10 which return an identity closest to *Stenotrophomonas rhizophila* DSM 14405 only BRP10 could solubilise tricalcium phosphate. Two isolates, BRP4 and BMC6 were found to possess just one of the five parameters tested namely the ability to inhibit the growth of *R. solani*. However two isolates, BRP48 and LAM2, were found to possess all five parameters tested and both were identified as *Pseudomonas helmanticensis*. Of the remainder, five isolates had a score of four, nine isolates had a score of three and the remaining three isolates had a score of two. The commercially available isolate *B. amyloliquifaciens* FZB24® was found to possess two parameters, NH_3 production and growth inhibition of *R. solani*.

Table 2. Biochemical characterisation of bacterial isolates for five commonly-reported PGP activities.

Genus	Isolate	$Ca_3(PO_4)_2$ Solubilisation	Siderophore Production	HCN	NH_3	Growth inhibition of *R. solani*	Score
Achromobacter	BRP1	+	-	+	+	+	**4**
Stenotrophomonas	BRP4	-	-	-	-	+	**1**
Stenotrophomonas	BRP10	+	-	-	-	+	**2**
Pseudomonas	BRP14	-	+	-	+	+	**3**
Achromobacter	BRP18	-	-	-	+	+	**2**
Glutamicibacter	BRP37	-	+	-	+	+	**3**
Pseudomonas	BRP48	+	+	+	+	+	**5**
Achromobacter	BMC3^	-	-	-	+	+	**2**
Pseudomonas	BMC4^	-	+	-	+	+	**3**
Lysinibacillus	BMC6^	-	-	-	-	+	**1**
Bacillus	BMC10^	+	+	-	+	+	**4**
Bacillus	BMC11^	+	+	-	+	+	**4**
Pseudomonas	LAM2^	+	+	+	+	+	**5**
Bacillus	LAM7^	+	+	-	+	+	**4**
Serratia	LAM10^	-	+	-	+	+	**3**
Ochrobactrum	LAC1^	+	-	-	+	+	**3**
Serratia	LAC2^	+	+	-	+	+	**4**
Micrococcus	LAC6^	+	+	-	+	-	**3**
Rhodococcus	BRQ6	+	+	-	+	-	**3**
Bacillus	BRQ10	-	+	-	+	+	**3**
Pseudomonas	BRQ16	+	-	+	+	-	**3**
Bacillus	FZB24	-	-	-	+	+	**2**

Biochemical characterisation of isolates. Five PGP parameters were investigated, positive activity is indicated by (+) and negative by (-). Negative activity in growth-inhibition of *R. solani* is defined as being less than that of the inhibitory activity

of *B. amyloliquifaciens* FZB24®. Isolates denoted with (^) were recovered from soils associated with organic cropping regimes.

Of the 21 sequenced isolates, 12 displayed the ability to solubilise tricalcium phosphate (Fig. 2) (Table 3). The most effective of these isolates was BRP48 (*Pseudomonas helmanticensis*), a strain which was first identified from forest soil in Northern Spain and has been previously shown to be an effective phosphate solubiliser (Ramírez-Bahena et al. 2014). The most effective phosphate solubilising isolates were mainly found in the *Pseudomonas* (BRP48, LAM2), *Serratia* (LAC2, LAM7) and *Achromobacter* (BRP1) genera. The least effective isolates from this analysis were found in both *Stenotrophomonas* (BRP 10) and *Bacillus* (BMC10, BMC11), displaying a clear distinction among genera in the ability to solubilise tricalcium phosphate. Phosphate solubilisation has been reported previously in *B. amyloliquefaciens* FZB24® however in our study there was negligible evidence for this using our methodology, with ambiguous clearance around the sample stab-point in solid NPRIB media which was difficult to distinguish from the shadowing of the stab-point itself (Fig. 3).

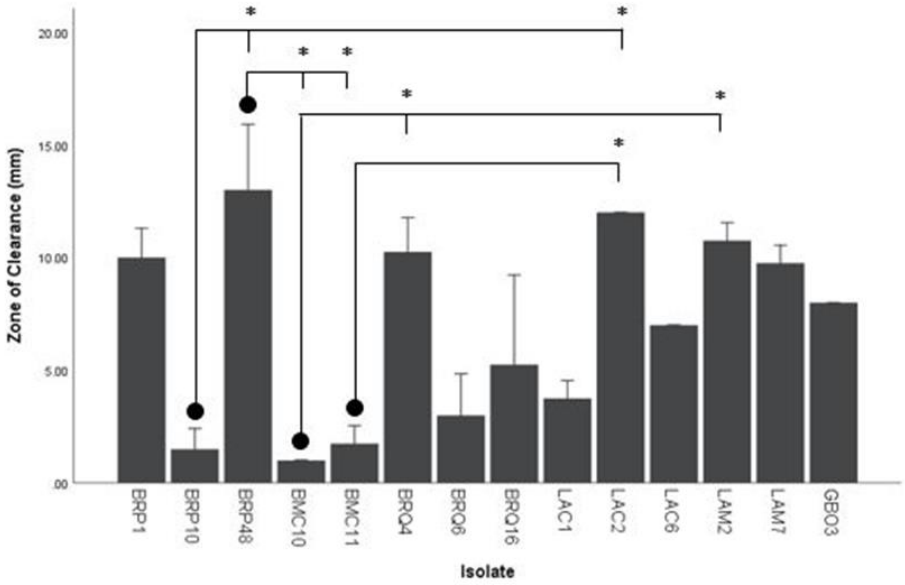


Fig. 2. Phosphate solubilisation activity of 13 bacterial isolates from both organic and conventional potato crop sites and *B. subtilis* GB03.Error bars represent confidence interval of the mean (95%) n=4. Isolate (GB03) used as control is *Bacillus subtilis* GB03. Significant differences ($p < 0.05$) are displayed by (*) between isolates with rooted columns displayed with a black circle and columns under respective branches. Pairwise comparisons with statistically significant values have been adjusted using the Bonferroni correction for multiple tests.

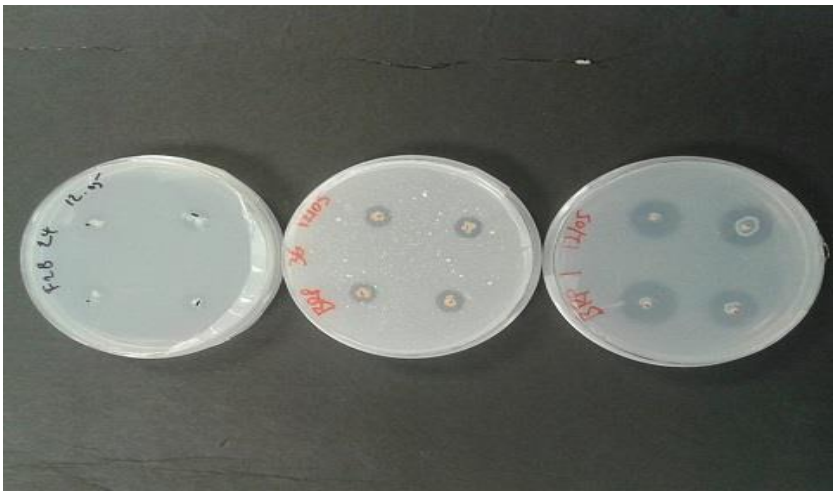


Fig 3. Phosphate solubilisation of NBRIP media by *B. amyloliquifaciens* FZB24® (left) and selected potato soil bacterial isolates (middle and right). Zones of clearance around colonies show active solubilisation of tricalcium phosphate.

Table 3. Tricalcium phosphate solubilisation capabilities of bacterial isolates from both organic and conventional potato crop sites.

Crop Site	Cultivar	Number of Isolates	Number of isolates capable of $Ca_3(PO_4)_2$ solubilisation
Barryroe	Premiere	7	3
	British Queen	3	2
Liss Ard^	Maris Bard	3	2
	Colleen	3	3
Ballymaloe Cookery School^	Colleen	5	2

Sites denoted with (^) indicate organic cropping regimes.

The isolates selected for identification by 16S rRNA sequence analysis from this cohort were chosen largely for their ability to inhibit fungal growth at a greater efficiency than the commercially available and well-characterised PGPR, *B. amyloliquefaciens* FZB24® (Fig. 4), as part of a larger study into the effect of BVCs on plant growth . However, three isolates displaying a weakened ability to inhibit fungal growth were also selected to determine if fungal growth-inhibition may be due in particular to a bacterial genus and/or species-specific effect in Irish potato soils. The most effective isolates overall were from the conventional site BRP4, BRP14 and BRP18, members of *Stenotrophomonas*, *Pseudomonas* and *Achromobacter* respectively with *R. solani* inhibition rates of 47.34%, 47.19% and 43.26%. From the organic sites the LAM isolates were the most efficient with LAM10 (*Serratia*) at 39.44% whereas the LAC isolates were the least efficient with LAC6 (*Micrococcus*), displaying an inhibition rate of 16.92%

(Fig. 5). A one way ANOVA did not display any significant differences between isolates ($p < 0.05$).

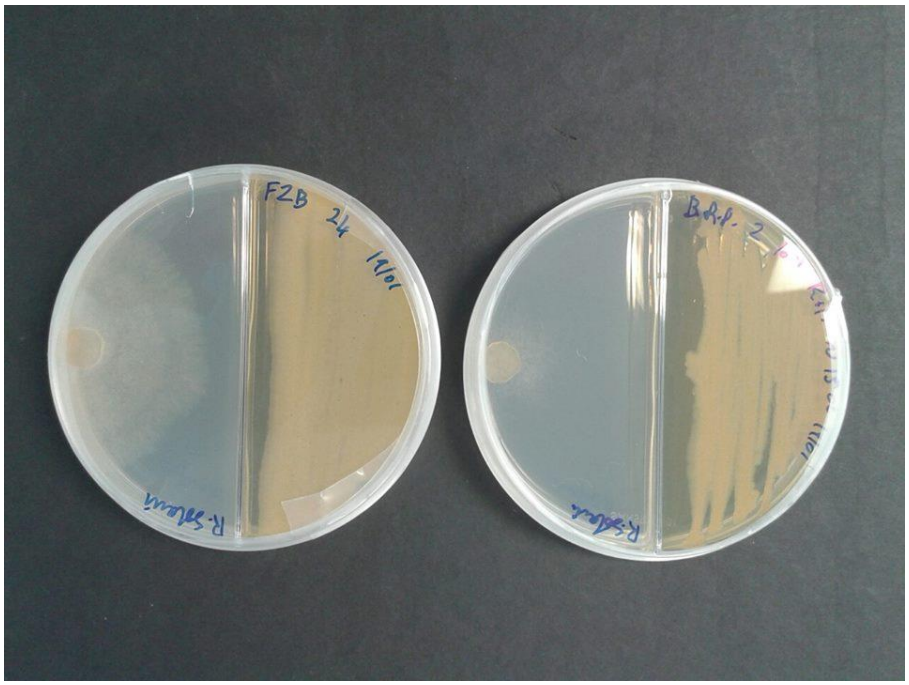


Fig. 4. Fungal growth inhibiton of *R. solani* proliferating from fungal plugs (left chamber of I-plates) mediated by BVCs released by selected bacterial isolates (right chamber of I-plates) in I-plate experimental analyses.

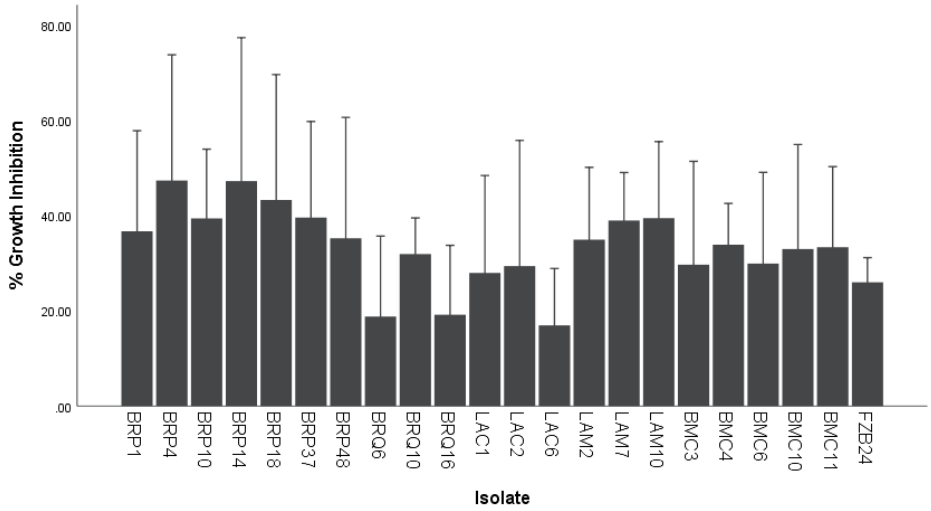


Fig. 5. Fungal growth-inhibition of *R. solani* mediated by bacterial volatile organic compounds produced by 22 isolates.Values are presented as percentage inhibition of fungal growth. Error bars represent confidence interval of the mean (95%), n=5. *B. amyloliquifaciens* FZB24® was used as a positive control as it is a PGPR which has been shown to have antifungal BVC activity (Velivelli et al. 2011).

Discussion:

Potato is an economically important crop both in Ireland and around the world. Historically, potato is closely associated with Ireland through an unprecedented decimation of the countries staple potato crop from 1845-49. During this period approximately one million people died from starvation and a further one million emigrated across the globe. In 2018, Ireland produced 273,000 tonnes of potato worth an estimated €85 million at the farm gate (CSO, 2019). Ireland currently has the third highest rate of CO_2 production per capita in the European Union and in 2017 the agricultural sector was responsible for 33% of all greenhouse gas emissions in Ireland (SEAI, 2018). As part of Ireland's commitment to its reduction in greenhouse gas emissions through the implementation of the 2015 Paris agreement on climate action both the Irish agricultural sector needs to reduce its carbon footprint. Given the historic context of Irish potato soils and the responsibilities that Ireland, and indeed the world, are facing in the context of climate change mitigation, alternative solutions to synthetic fertilisers and PPP are of paramount importance. This study is a preliminary investigation of Irish potato soils for the presence of PGPR, which can form part of this solution. Across all sites tested there was a considerable amount of bacterial diversity, members of *Bacillus*, *Pseudomonas* and *Achromobacter* were isolated from both organic and conventional crops and in some cases, through 16S rRNA analysis, it appears identical isolates are present in different sites/cultivars, however to confirm this a greater depth of sequencing is needed such

as whole-genome sequencing or, shallow shotgun sequencing for rhizobial community-wide analysis. This technique has proved useful for reasonably accurate profiling of the human gut bacterial community and may be a powerful and cost-effective tool for soil microbiome analyses in the near future as reference genomes for environmental isolates improve (Hillmann et al. 2018). For a more accurate identification of environmental isolates whole-genome sequencing can be employed, however this can be a budget-limiting factor for many studies due to the relatively high cost as was the case in this study, as sequencing technology advances and costs reduce this may become common place in the near future. The isolates BRP4 and BRP10 were identified as *Stenotrophomonas rhizophila* but were not equally capable of phosphate solublisation, only BRP10 showed a minimal level of phosphate solubilisation. It has been reported in previous studies that *S. rhizophila* strains solubilise tricalcium phosphate in different degrees (Afzal et al. 2015; Heredia-Acuña et al. 2019). Phosphate solubilisation was most efficient in the genera *Pseudomonas*, *Serratia* and *Achromobacter* and this is evidenced by many previous studies regarding the ability of members of these genera to efficiently solubilise inorganic phosphates (Martin et al. 2007; Devi et al. 2013; Pande et al. 2017). Given that world phosphate supplies are a finite resource, and P inputs are a significant factor in current agricultural practice it is crucial for agriculture to make the most efficient use of this resource, PGPR capable of phosphate solubilisation represent an excellent co-application to minimise P input and maximise

the P-use efficiency of modern agriculture (Gaswami et al. 2016; Li et al. 2018; Ben Zinab et al. 2020; Mpanga et al. 2020). Siderophore production was observed in 13 isolates particularly among the *Pseudomonas* sp. isolates, interestingly, of the five members of this genus, only one was observed not to produce siderophores; BRQ16. This isolate has highest relatedness to *Pseudomonas fragi* which is known to scavenge iron from the surrounding environment rather than produce its own siderophores (Champomier-Vergès et al. 1996). The isolates with highest relatedness to *S. rhizophila* also did not produce siderophores in our analysis, this could be due to the ability of *Stenotrophomonas* species to efficiently capture siderophores from their surrounding environment to aid bacterial cell growth and particularly, as a competitive mechanism to inhibit the growth of phytopathogens such as the fungal genus *Ustilago* which produces the siderophore ferrichome (Jurkevitch et al. 1992; Ardon et al. 1997; Ryan et al. 2009). Not only can *S. rhizophila* form part of an integrated pest management approach to fungal phytopathogens, but it has also been reported that biocidal activity against root-knot nematodes such as *Meloidogyne incognita*, can be employed under both greenhouse and microplot scenarios with *Cynodon* spp. L. (bermudagrass) (Groover et al. 2020).

Further studies have highlighted the genus *Stenotrophomonas* as having both direct and indirect PGP properties, in particular *Stenotrophomonas rhizophila* (Wolf et al. 2002; Kai et al. 2007).

Interestingly, Schmidt et al. (2012) reported that *S. rhizophila* DSM 14405 can indirectly promote plant growth via the alteration of the fungal community in the rhizosphere of tomato (*Solanum lycopersicum*) and sweet pepper (*Capsicum annuum*) with a particularly strong effect on the tomato rhizosphere. In non-sterile soil, the PGP effect of *S. rhizophila* was greater than that of a gnotobiotic growth system, indicating that the indirect growth effect observed is due to a competitive inhibition of growth in phytopathogens and deleterious microbes (Schmidt et al. 2012). The numerous PGP activities displayed by *S. rhizophila* have been demonstrated to also protect plant hosts from abiotic stressors such as highly salinated soils (Egamberdieva et al. 2011). The effect of salinity and root exudates on the transcriptome, and the subsequent production of both phytoprotective and self-protective metabolites of *S. rhizophila* displayed that in terms of bacterial defence against salt stress, the negative regulation of genes coding for flagella-related proteins and the upregulation of genes associated with biofilm formation and biosynthesis of alginate were identified as the main mechanism employed by *S. rhizophila* to cope with salt shock (Alavi et al. 2013). Similar expression profiles were observed when the bacterium was exposed to root exudates of oilseed rape which are consistent with the initiation of plant colonisation, this is not surprising as colonisation of the host can help protect against adverse abiotic conditions. The reciprocal bacterial contribution toward survival of the host is evidenced by the increased production of spermidine. This bioactive

compound offers stress protection to the root system and is a well-known regulator of plant growth with a critical role observed in plant embryo development (Imai et al. 2004) and also affects bacterial biofilm formation (McGinnis et al. 2009). Therefore spermidine could play a role in the enhanced colonisation of the plant host by *S. rhizophila* while also directly benefitting the health status of the plant host (Alavi et al. 2013).

Quorum sensing (QS) is an essential system for communication among many bacteria, including plant pathogenic bacteria to coordinate the efficient virulence effect toward their respective hosts (von Bodman et al. 2003). Disruption of this communication system can be exploited for the biocontrol of pathogenic organisms. From our study we have identified two isolates from Irish potato soils which have been shown previously to have quorum quenching (QQ) capability. Both *Bacillus toyonensis* and *Rhodococcus erythropolis* isolated from organic and conventional sites respectively have demonstrated their suitability as potential biocontrol PGPR that may utilise QQ activity. The well-known insecticidal bacterium *Bacillus thuringiensis,* a close relative of *B. toyonensis,* was shown to possess a widely conserved N-acylhomoserine lactone-lactonase (AHLase) among *Bacillus* strains, this enzyme disrupts QS in *Pectobacterium carotavorum* (formerly *Erwinina carotovora*) by hydrolysing the homoserine lactone ring portion of N-acylhomoserine lactone. *B. toyonensis* and *B. thuringiensis* belong to a larger group of *Bacillus* strains with close

phylogenetic relationships, this group is termed *Bacillus cereus sensu lato* and occupies a large ecological diversity (Bazinet, 2017; Fayad et al. 2019). Genome analysis of *B. toyonensis* revealed a key gene, *aiiA*, which encodes an AHLase, presumably responsible for the ability to supress QS in gram-negative bacteria (Lopes et al. 2015; Lopes et al. 2017). Pre-treatment of potato slices with *B. thuringiensis* B23 suppressed *P. carotavorum* infection with no maceration symptoms visible after 24 hours (Dong et al. 2004). The effect of Quorum-related biocontrol by *R. erythropolis* has been extensively investigated. Uroz et al. first described the bioprotective QQ activity of *R. erythropolis* in planta using potato tubers which were exposed to *P. carotavorum* (Uroz et al. 2003). Barbey et al. found further evidence of the biocontrol potential of *R. erythropolis* by demonstrating that the γ-lactone catabolic pathway of this bacterium can disrupt the communication between cells of *Pectobacterium atrosepticum*, another pathogen of potato.

Previous studies to this had indicated that pathogen invasion was the trigger for QQ-mediated biocontrol, however in this instance the act of pathogen communication was enough to trigger a γ-lactone catabolic pathway-mediated QQ response. The key enzyme in this pathway, QsdA, was shown to be able to protect potato tubers against *P. atrosepticum* when heterologously expressed by *E. coli* equal to that of the wild-type *R. erythropolis* strain. Furthermore, deletion of the *qsdA* gene in *R. erythropolis* resulted in a significantly higher degree of

tissue maceration of potato from three days post-inoculation to the end of the experiment with *P. atrosepticum* compared to that of the QsdA-expressing parental strain used in co-infections, interestingly, deletion of the *qsdA* gene did not entirely abolish the biocontrol activity (Barbey et al. 2013). This may be due to the ability of *Rhodococci* to express multiple homologs of catabolic enzymes (van der Geize and Dijkhuizen, 2004) which may work in conjunction with or independently of QsdA or inactivation of this pathway may induce the activation of an alternative metabolic pathway (Kaufmann et al. 2005).

The production of volatile organic compounds and their effect on microbe-microbe and plant-microbe interactions has been a significant area of research since the ground-breaking publication by Ryu et al. (2004) investigating the effects of bacterial VOCs (BVCs) on the resistome of *Arabidopsis*. The role of BVCs as a direct treatment or alternatively as progenitors for chemical analogues will play an important part of sustainable agriculture in the future (Báez-Fallejo et al. 2020; Torres et al. 2020). In our study all the bacterial isolates tested had some level of VOC-mediated growth-inhibition of *R. solani*, some such as BRP4 (*Stenotrophomonas*) and BRP14 (*Pseudomonas*) were much more effective than others such as LAC6 (*Micrococcus*) and BRQ6 (*Rhodococcus*). It is interesting to note that the two members of the Actinobacteria were among the least effective at VOC-mediated growth-inhibition of *R. solani*. While there has been studies on the antibiotic effect of Actinobacterial VOCs against phytopathogenic

bacteria (Choudoir et al. 2019; Avalos et al. 2018), there is little to no information on the effect of Actinobacterial VOCs against *R. solani*. Therefore this may be a tentative indication of a niche within the rhizosphere which Actinobacteria do not necessarily exploit in the presence of this fungal phytopathogen, this is worthy of further investigation.

The observed bacterial diversity between potato crops growing in close proximity to each other at the same site, particularly Barryroe, may be indicative of different cultivars recruiting different bacterial symbionts from the surrounding bulk soil via modulation of root exudate constituents at the root/soil interface to exploit different ecobacteriological niches (Lundberg et al. 2012). Mutual overlap of bacterial isolates, namely those closest in identity to *Bacillus mycoides* and *Pseudomonas helmanticensis* was observed between Liss Ard and Barryroe. Additionally with *Achromobacter marplatensis* between Ballymaloe Cookery School and Barryroe, each organic and conventional sites respectively. This could indicate that perhaps there may be a 'core microbiome' among Irish potato soils, at least where soil characteristics are the same or similar across sampling sites.

The fact that many of the isolates from this study have been identified in other potato-related studies may indeed be an indication that potato recruits a 'core microbiome' consisting of a bacterial consortia responsible for protection against phytopathogens and enhanced nutrient acquisition. Evidence for a core microbiome has been

demonstrated to exist in the potato rhizosphere of the high Andes irrespective of the respective crop site and the stage of growth (Pfeiffer et al. 2017). Additionally, the core and differentially abundant bacterial taxa in *Brassica napus* has recently been reported over a dynamic temporal, spatial and plant genotypical set of parameters, *B. napus* appears to recruit a core microbiome across soil type and environment with *Bradyrhizobiome, Arthrobacter* and *Stenotrophomonas* appearing to be the core members with potential for beneficial PGP activities (Taye et al. 2020). One isolate from our study, (*Pseudomonas helmanticensis*) was capable of all five PGP biochemical activities tested and was found in both organic and conventional soils, suggesting that this isolate may be an effective biological input for sustainable agriculture in the future for both cropping regimes. Indeed the overlap observed in some bacterial isolates obtained from both cropping regimes may not be entirely surprising as it has been demonstrated that only around 2% of bacterial phylotypes (~500 phylotypes) make up about half of all bacterial soil communities isolated from 237 locations from all six continents (Delgado-Baquerizo et al. 2018). Interestingly, it has recently been reported that the microbiome between organic and conventional sites is almost identical and can remain robustly stable across soil types which have similar parameters even when inputs such as phosphorus are applied in varying degrees. Using a metagenomics approach Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes and Bacteroidetes were identified as the dominant phyla present in both organic and conventional farming systems (Armalyté et al. 2019). The

multifactorial PGP properties displayed by many of the isolates in this study may be tailored to suit specific crops and/or soil parameters. Repeated field application of these native isolates in an Irish context will need to be carried out to determine their potential to cope with the ever changing climate of Northwestern Europe. Nonetheless, results from the bacterial isolates of this study corroborate those of previous studies in many cases and offer a preliminary snapshot into the rhizospheric microbiome of Irish potato soils. As we mine the soil worldwide for microbial solutions to our climate crisis coupled with the possibility of a knock-on food crisis, the isolates identified in this study can be added to the knowledge base of what bacteria may be suitable to augment current and future agricultural practice for enhanced potato production as we face into a warming and ecologically uncertain future.

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References:

Afzal IM, Shinwari ZK, Iqrar I (2015) Selective isolation and characterization of agriculturally beneficial endophytic bacteria from wild hemp using canola. Pak J Bot 47:1999-2008

Agamennone V, van Straalen J, Brouwer A, de Boer TE, Hensbergen PJ, Zaagman N, Braster M, van Straalen NM, Roelofs D, Janssens TK (2019) Genome annotation and antimicrobial properties of *Bacillus toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. Entomol Exp Appl 167:269-285

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173-81

Alavi P, Starcher MR, Zachow C, Müller H, Berg G (2013) Root-microbe systems: the effect and mode of interaction of Stress Protecting Agent (SPA) *Stenotrophomonas rhizophila* DSM14405^{T}. Front Plant Sci 4:141

Ardon O, Weizman H, Libman J, Shanzer A, Chen Y, Hadar Y (1997) Iron uptake in *Ustilago maydis*: studies with fluorescent ferrichrome analogues. Microbiology 143:3625-31

Armalytė J, Skerniškytė J, Bakienė E, Krasauskas R, Šiugždinienė R, Kareivienė V, Kerzienė S, Klimienė I, Sužiedėlienė E, Ružauskas M (2019) Microbial Diversity and Antimicrobial Resistance Profile in Microbiota From Soils of Conventional and Organic Farming Systems. Front Microbiol 10:892

Aslam F, Ali B (2018) Halotolerant Bacterial Diversity Associated with *Suaeda fruticosa* (L.) Forssk. Improved Growth of Maize under Salinity Stress. Agronomy 8:131

Avalos M, van Wezel GP, Raaijmakers JM, Garbeva P (2018) Healthy scents: microbial volatiles as new frontier in antibiotic research? Curr Opin Microbiol 45:84-91.

Barbey C, Crépin A, Bergeau D, Ouchiha A, Mijouin L, Taupin L, Orange N, Feuilloley M, Dufour A, Burini JF, Latour X (2013) In planta biocontrol of *Pectobacterium atrosepticum* by *Rhodococcus erythropolis* involves silencing of pathogen communication by the rhodococcal gamma-lactone catabolic pathway. PLoS One 8:e66642

Báez-Vallejo N, Camarena-Pozos DA, Monribot-Villanueva JL, Ramírez-Vázquez M, Carrión-Villarnovo GL, Guerrero-Analco JA, Partida-Martínez LP, Reverchon F (2020) Forest tree associated bacteria for potential biological control of *Fusarium solani* and of

Fusarium kuroshium, causal agent of *Fusarium* dieback. Microbiol Res 235:126440.

Bazinet AL (2017) Pan-genome and phylogeny of *Bacillus cereus* sensu *lato*. BMC Evol Biol 17:176

Behera P, Mahapatra M, Seuylemezian A, Vaishampayan P, Ramana VV, Joseph N, Joshi A, Shouche YS, Suar M, Pattnaik AK, Rastogi G (2018) Taxonomic description and draft genome of *Pseudomonas sediminis* sp. nov., isolated from the rhizospheric sediment of *Phragmites karka*. J Microbiol 56:458-466

Ben Zineb A, Trabelsi D, Ayachi I, Barhoumi F, Aroca R, Mhamdi R (2020) Inoculation with Elite Strains of Phosphate-Solubilizing Bacteria Enhances the Effectiveness of Fertilization with Rock Phosphates. Geomicrobiol J 37:22-30

Billah, M., Khan, M., Bano, A., Hassan, T. U., Munir, A., & Gurmani, A. R. (2019). Phosphorus and phosphate solubilizing bacteria: Keys for sustainable agriculture. *Geomicrobiology Journal*, *36*(10), 904-916.

Cappuccino JC, Sherman, N (1992) Negative staining. In: Microbiology: A Laboratory Manual, 3rd ed., Benjamin Cummings, New York, pp. 125–179

Champomier-Vergès MC, Stintzi A, Meyer JM (1996) Acquisition of iron by the non-siderophore-producing *Pseudomonas fragi*. Microbiology 142:1191-1199

Choudoir M, Rossabi S, Gebert M, Helmig D, Fierer N (2019) A phylogenetic and functional perspective on volatile organic compound production by actinobacteria. MSystems 4:e00295-18.

Cirou A, Raffoux A, Diallo S, Latour X, Dessaux Y, Faure D (2011) Gamma-caprolactone stimulates growth of quorum-quenching *Rhodococcus* populations in a large-scale hydroponic system for culturing *Solanum tuberosum*. Res Microbiol 162:945-950

CSO (2019) AQA04: Crop Yield and Production by Type of Crop, Year and Statistic. Central Statistics Office, Skehard Road, Cork, Ireland

Daly DH, Velivelli SLS, Prestwich BD (2017) The Role of Soil Microbes in Crop Biofortification. In: Meena V, Mishra P, Bisht J, Pattanayak A (eds) Agriculturally Important Microbes for Sustainable Agriculture. Springer, Singapore, pp. 333-356

Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge DJ, Bardgett RD, Maestre FT, Singh BK, Fierer N (2018)

A global atlas of the dominant bacteria found in soil. Science 359:320-325

Devaux, A., Goffart, J. P., Kromann, P., Andrade-Piedra, J., Polar, V., & Hareau, G. (2021). The Potato of the Future: Opportunities and Challenges in Sustainable Agri-food Systems. Potato Res 64:681-720.

Devi U, Khatri I, Kumar N, Kumar L, Sharma D, Subramanian S, Saini AK (2013) Draft genome sequence of a plant growth-promoting rhizobacterium, *Serratia fonticola* strain AU-P3 (3). Genome Announc 1:e00946-13

Dong YH, Zhang XF, Xu JL, Zhang LH (2004) Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Appl Environ Microbiol 70:954-60

Egamberdieva D, Kucharova Z, Davranov K, Berg G, Makarova N, Azarova T, Chebotar V, Tikhonovich I, Kamilova F, Validov SZ, Lugtenberg B (2011) Bacteria able to control foot and root rot and to promote growth of cucumber in salinated soils. Biol Fertil Soils 47:197-205

Elo S, Maunuksela L, Salkinoja-Salonen M, Smolander A, Haahtela K (2000) Humus bacteria of Norway spruce stands: plant growth promoting properties and birch, red fescue and alder colonizing capacity. FEMS Microbiol Ecol 31:143-152

Fayad N, Awad MK, Mahillon J (2019) Diversity of *Bacillus cereus* sensu *lato* mobilome. BMC Genomics 20:436

Fotheringham AS, Kelly MH, Charlton M (2013) The demographic impacts of the Irish famine: towards a greater geographical understanding. Trans Inst Br Geogr 38:221-237

García-Fraile P, Chudíčková M, Benada O, Pikula J, Kolařík M (2015) *Serratia myotis* sp. nov. and *Serratia vespertilionis* sp. nov., isolated from bats hibernating in caves. Int J Syst Evol Microbiol 65:90-94

Goswami D, Thakker JN, Dhandhukia PC (2016) Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric 2:1127500

Ghyselinck J, Velivelli SL, Heylen K, O'Herlihy E, Franco J, Rojas M, De Vos P, Prestwich BD (2013) Bioprospecting in potato fields in the Central Andean Highlands: screening of rhizobacteria for plant growth-promoting properties. Syst Appl Microbiol 36:116-127

Gomila M, Tvrzova L, Teshim A, González Escalona N, Zdráhal Z, Šedo O, González JF, Bennasar A, Moore ER, Lalucat J, Murialdo SE

(2011) *Achromobacter marplatensis* sp. nov., isolated from a pentachlorophenol contaminated soil. Int J Syst Evol Microbiol 61

Groover W, Held D, Lawrence K, Carson K (2020) Plant growth-promoting rhizobacteria: a novel management strategy for *Meloidogyne incognita* on turfgrass. Pest Manag Sci 76:3127-3138.

Haverkort AJ, Struik PC, Visser RG, Jacobsen EJ (2009) Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. Potato Res 52:249-264

Heredia-Acuña C, Almaraz-Suarez JJ, Arteaga-Garibay R, Ferrera-Cerrato R, Pineda-Mendoza DY (2019) Isolation, characterization and effect of plant-growth-promoting rhizobacteria on pine seedlings (*Pinus pseudostrobus* Lindl.). J For Res 30:1727-1734

Hillmann B, Al-Ghalith GA, Shields-Cutler RR, Zhu Q, Gohl DM, Beckman KB, Knight R, Knights D (2018) Evaluating the information content of shallow shotgun metagenomics. MSystems 3:e00069-18

Huang JS, Peng YH, Chung KR, Huang JW (2018) Suppressive efficacy of volatile compounds produced by *Bacillus mycoides* on damping-off pathogens of cabbage seedlings. J Agric Sci 156:795-809

Hunziker L, Bönisch D, Groenhagen U, Bailly A, Schulz S, Weisskopf L (2015) *Pseudomonas* strains naturally associated with potato plants produce volatiles with high potential for inhibition of *Phytophthora infestans*. Appl Environ Microbiol 81:821-830

Imai A, Matsuyama T, Hanzawa Y, Akiyama T, Tamaoki M, Saji H, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S (2004) Spermidine synthase genes are essential for survival of *Arabidopsis*. Plant Physiol 135:1565-1573

Jung BK, Khan AR, Hong SJ, Park GS, Park YJ, Park CE, Jeon HJ, Lee SE, Shin JH (2017) Genomic and phenotypic analyses of *Serratia fonticola* strain GS2: a rhizobacterium isolated from sesame rhizosphere that promotes plant growth and produces N-acyl homoserine lactone. J Biotech 241:158-162

Jurkevitch E, Hadar Y, Chen Y (1992) Differential siderophore utilization and iron uptake by soil and rhizosphere bacteria. Appl Environ Microbiol 58:119-124

Kai M, Effmert U, Berg G, Piechulla B (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Arch Microbiol 187:351-360

Kämpfer P, Sessitsch A, Schloter M, Huber B, Busse HJ, Scholz HC (2008) *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum*

thiophenivorans sp. nov., isolated from the environment. Int J Syst Evol Microbiol 58:1426-1431

Kanetis L, Tsimouris D, Christoforou M (2016) Characterization of *Rhizoctonia solani* Associated with Black Scurf in Cyprus. Plant Dis 100:1591-1598

Kaufmann GF, Sartorio R, Lee SH, Rogers CJ, Meijler MM, Moss JA, Clapham B, Brogan AP, Dickerson TJ, Janda KD (2005) Revisiting quorum sensing: discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones. Proc Natl Acad Sci U S A 102:309-314

Kloepper JW, Schroth MN (1978) Plant growth promoting rhizobacteria on radishes. In: Proceedings of the 4th international conference on plant pathogenic bacteria, Angers, France, vol 2, pp 879–882

Lafi FF, Ramirez-Prado JS, Alam I, Bajic VB, Hirt H, Saad MM (2017) Draft genome sequence of plant growth–promoting *Micrococcus luteus* strain K39 isolated from *Cyperus conglomeratus* in Saudi Arabia. Genome Announc 5:e01520-16

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955-9.

Leff B, Ramankutty N, Foley JA (2004) Geographic distribution of major crops across the world. Global Biogeochem Cy 18(1)

Lettice EP, Jones PW (2017) Bacterial functional diversity in Irish potato field soil, as determined by community-level physiological profiling. Biol Environ doi:10.3318/ BIOE.2017.10

Li B, Boiarkina I, Young B, Yu W, Singhal N (2018) Prediction of future phosphate rock: a demand based model. J Environ Inf 1:41-53

Lopes RB, de Oliveira Costa LE, Vanetti MC, de Araújo EF, de Queiroz MV (2015) Endophytic bacteria isolated from common bean (*Phaseolus vulgaris*) exhibiting high variability showed antimicrobial activity and quorum sensing inhibition. Curr Microbiol 71:509-516

Lopes R, Cerdeira L, Tavares GS, Ruiz JC, Blom J, Horácio EC, Mantovani HC, de Queiroz MV (2017) Genome analysis reveals insights of the endophytic *Bacillus toyonensis* BAC3151 as a potentially novel agent for biocontrol of plant pathogens. World J Microb Biot 33:185

Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, Tremblay J, Engelbrektson A, Kunin V, Del Rio TG, Edgar RC

(2012) Defining the core *Arabidopsis thaliana* root microbiome. Nature 488:86-90

Lucy M, Reed E, Glick BR (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie van leeuwenhoek 86:1-25

McGinnis MW, Parker ZM, Walter NE, Rutkovsky AC, Cartaya-Marin C, Karatan E (2009) Spermidine regulates *Vibrio cholerae* biofilm formation via transport and signaling pathways. FEMS Microbiol Lett 299:166-174

Martin L, Velázquez E, Rivas R, Mateos PF, Martínez-Molina E, Rodríguez-Barrueco C, Peix A (2007) Effect of inoculation with a strain of *Pseudomonas fragi* in the growth and phosphorous content of strawberry plants. In: First International Meeting on Microbial Phosphate Solubilization. Springer, Dordrecht pp. 309-315

Mpanga IK, Ludewig U, Dapaah HK, Neumann G (2020) Acquisition of Rock Phosphate by combined application of Ammonium fertilizers and *Bacillus amyloliquefaciens* FZB42 in Maize as affected by Soil pH. J Appl Microbiol doi:10.1111/jam.14654

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265-70

Oka, Y., Chet, I., & Spiegel, Y. (1993). Control of the rootknot nematode *Meloidogyne javanica* by *Bacillus cereus*. Biocontrol Sci Technol 3:115-126.

Pande A, Pandey P, Mehra S, Singh M, Kaushik S (2017) Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. J Genet Eng Biotechnol 15:379-391

Pérez-Miranda S, Cabirol N, George-Téllez R, Zamudio-Rivera LS, Fernández FJ (2007) O-CAS, a fast and universal method for siderophore detection. J Microbiol Methods 70:127-31

Pfeiffer S, Mitter B, Oswald A, Schloter-Hai B, Schloter M, Declerck S, Sessitsch A (2017) Rhizosphere microbiomes of potato cultivated in the High Andes show stable and dynamic core microbiomes with different responses to plant development. FEMS Microbiol Ecol 93:fiw242

Radzki, W., Mañero, F. G., Algar, E., García, J. L., García-Villaraco, A., & Solano, B. R. (2013). Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie Van Leeuwenhoek 104:321-330.

Ramírez-Bahena MH, Cuesta MJ, Flores-Félix JD, Mulas R, Rivas R, Castro-Pinto J, Brañas J, Mulas D, González-Andrés F, Velázquez E, Peix A (2014) *Pseudomonas helmanticensis* sp. nov., isolated from forest soil. Int J Syst Evol Microbiol 64:2338-2345

Raza FA, Faisal M (2013) Growth promotion of maize by desiccation tolerant *Micrococcus luteus*-chp37 isolated from Cholistan desert, Pakistan. Aust J Crop Sci 7:1693

Ryan RP, Monchy S, Cardinale M, Taghavi S, Crossman L, Avison MB, Berg G, Van Der Lelie D, Dow JM (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat Rev Microbiol 7:514-25

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol 134:1017-26

Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J 3:283-300

Sang MK, Kim EN, Han GD, Kwack MS, Jeun YC, Kim KD (2014) Priming-mediated systemic resistance in cucumber induced by *Pseudomonas azotoformans* GC-B19 and *Paenibacillus elgii* MM-B22 against *Colletotrichum orbiculare*. Phytopathology 104:834-842

Savary S, Willocquet L, Pethybridge SJ, Esker P, McRoberts N, Nelson A (2019) The global burden of pathogens and pests on major food crops. Nat Ecol 3:430

Sayyed, R. Z., Chincholkar, S. B., Reddy, M. S., Gangurde, N. S., & Patel, P. R. (2013). Siderophore producing PGPR for crop nutrition and phytopathogen suppression. In *Bacteria in agrobiology: disease management* (pp. 449-471). Springer, Berlin, Heidelberg.

Schmidt CS, Alavi M, Cardinale M, Müller H, Berg G (2012) *Stenotrophomonas rhizophila* DSM14405^T promotes plant growth probably by altering fungal communities in the rhizosphere. Biol Fertil Soils 48:947–960

SEAI (2018) Energy-Related CO_2 Emissions in Ireland 2005-2016. Sustainable Energy Authority of Ireland 2018 report.

Selvakumar G, Joshi P, Nazim S, Mishra P, Bisht J, Gupta H (2009) Phosphate solubilization and growth promotion by *Pseudomonas fragi* CS11RH1 (MTCC 8984), a psychrotolerant bacterium isolated from a high altitude Himalayan rhizosphere. Biologia 64:239-45

Shi Y, Li C, Yang H, Zhang T, Gao Y, Chu M, Zeng J, Lin Q, Li Y, Huo X, Lou K (2017) Colonization study of gfp-tagged *Achromobacter marplatensis* strain in sugar beet. J Microbiol 55:267-272

Siddikee MA, Chauhan PS, Anandham R, Han GH, Sa T (2010) Isolation, characterization, and use for plant growth promotion under salt stress, of ACC deaminase-producing halotolerant bacteria derived from coastal soil. J Microbiol Biotechnol 20:1577-1584

Smith P, Bustamante M, Ahammad H, Clark H, Dong H, Elsiddig EA, Haberl H, Harper R, House J, Jafari M, Masera O, Mbow C, Ravindranath NH, Rice CW, Robeldo Abad C, Romanovskaya A, Sperling F, Tubiello F (2014) Agriculture, Forestry and Other Land Use (AFOLU). In: Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Edenhofer O, Pichs-Madruga R, Sokona Y, Farahani E, Kadner S, Seyboth K, Adler A, Baum I, Brunner S, Eickemeier P, Kriemann B, Savolainen J, Schlömer S, von Stechow C, Zwickel T, Minx JC (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA

Suvega T, Arunkumar K (2019) Probiotic bacteria promote the growth of associating host (red seaweed, *Gracilaria edulis*) also synthesize antibacterial protein. Biocatal Agric Biotechnol 19:101136

Talley SM, Coley PD, Kursar TA (2002) The effects of weather on fungal abundance and richness among 25 communities in the Intermountain West. BMC ecology 2:7

Taye ZM, Helgason BL, Bell JK, Norris CE, Vail S, Robinson SJ, Parkin IA, Arcand M, Mamet S, Links MG, Dowhy T (2020) Core and Differentially Abundant Bacterial Taxa in the Rhizosphere of Field Grown *Brassica napus* Genotypes: Implications for Canola Breeding. Front Microbiol 10:3007

Torres M, Llamas I, Torres B, Toral L, Sampedro I, Béjar V (2020) Growth promotion on horticultural crops and antifungal activity of *Bacillus velezensis* XT1. Appl Soil Ecol 150:103453

Trivedi P, Pandey A, Sa T (2007) Chromate reducing and plant growth promoting activies of psychrotrophic *Rhodococcus erythropolis* MtCC 7905. J Basic Microbiol 47:513-517

Uroz S, D'Angelo-Picard C, Carlier A, Elasri M, Sicot C, Petit A, Oger P, Faure D, Dessaux Y (2003) Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. Microbiology 149:1981-1989

Vandamme PA, Peeters C, Inganäs E, Cnockaert M, Houf K, Spilker T, Moore ER, LiPuma JJ (2016) Taxonomic dissection of *Achromobacter denitrificans* Coenye et al. 2003 and proposal of *Achromobacter agilis* sp. nov., nom. rev., *Achromobacter pestifer* sp. nov., nom. rev., *Achromobacter kerstersii* sp. nov. and *Achromobacter deleyi* sp. nov. Int J Syst Evol Microbiology 66:3708-3717

van der Geize R, Dijkhuizen L (2004) Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications. Curr Opin Microbiol 7:255-261

Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A (2016) Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. Molecules 21:573

Velivelli SL, De Vos P, Kromann P, Declerck S, Prestwich, BD (2014) Biological control agents: from field to market, problems, and challenges. Trend Biotech 32:493-496

Velivelli S, O'Herlihy E, Janczura B, Doyle Prestwich B, Ghyselinck J, De Vos P (2011) Efficacy of Rhizobacteria on plant growth promotion and disease suppression in vitro. In: VII International Symposium on In Vitro Culture and Horticultural Breeding 961 pp. 525-532

von Bodman SB, Bauer WD, Coplin DL (2003) Quorum sensing in plant-pathogenic bacteria. Ann Rev Phytopath 41:455-482

Warshan D, Liaimer A, Pederson E, Kim SY, Shapiro N, Woyke T, Rasmussen U (2018) Genomic changes associated with the evolutionary transitions of *Nostoc* to a plant symbiont. Mol Biol Evol 35:1160-1175

Wei G, Kloepper JW, Tuzun S (1996) Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathology

Wolf A, Fritze A, Hagemann M, Berg G (2002) *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. Int J Syst Evol Microbiol 56:1937-1944

Yi Y, de Jong A, Frenzel E, Kuipers OP (2017) Comparative transcriptomics of *Bacillus mycoides* strains in response to potato-root exudates reveals different genetic adaptation of endophytic and soil isolates. Front Microbiol 8:1487

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: A taxonomically united database of 16S

rRNA and whole genome assemblies. Int J Syst Evol Microbiol. 67:1613-1617

Chapter 4

Volatile compounds from *Bacillus*, *Serratia* and *Pseudomonas* promote growth and alter the transcriptional landscape of *Solanum tuberosum* in a passively-ventilated growth system

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Abstract:

The interaction of an array of volatile organic compounds (VOCs) termed bacterial volatile compounds (BVCs) with plants is now a major area of study under the umbrella of plant-microbe interactions. Many growth systems have been developed to determine the nature of these interactions in vitro. However, each of these systems have their benefits and drawbacks with respect to one another and can greatly influence the end-point interpretation of the BVC effect on plant physiology. To address the need for novel growth systems in BVC-Plant interactions, our study investigated the use of a passively-ventilated growth system,

made possible via Microbox® growth chambers, to determine the effect of BVCs emitted by six bacterial isolates from the genera *Bacillus*, *Serratia* and *Pseudomonas*. Solid-phase microextraction GC/MS was utilized to determine the BVC profile of each bacterial isolate when cultured in three different growth media each with varying carbon content. 70 BVCs were identified in total, with alcohols and alkanes being the most abundant. When cultured in tryptic soy broth, all six isolates were capable of producing 2,5-dimethylpyrazine, however BVC emission associated with this media were deemed to have negative effects on plant growth. The two remaining media types, namely Methyl Red-Voges Proskeur (MR-VP) and Murashige and Skoog, were selected for bacterial growth in co-cultivation experiments with *Solanum tuberosum* L. cv. 'Golden Wonder'. The BVC emissions of *Bacillus* and *Serratia* isolates cultured on MR-VP induced alterations in the transcriptional landscape of potato across all treatments with 956 significantly differentially expressed genes. Our results indicate that this novel approach to determining BVC-mediated growth effects on plants is a viable addition to current methodologies and displayed differential growth-promotion based on bacterial isolate and the respective media type on which they were cultured. Surprisingly, genes associated with plant defense were often observed to be downregulated in our system whereas genes associated with photosynthesis and plant cell division were upregulated.

Introduction:

The relationship between plants and their consortia of microbial associates is recognised as one of the most ancient relationships on Earth. Today, we have the opportunity to exploit this relationship which can benefit the environment and thus, society as a whole. Numerous studies have demonstrated the ability of plant-associated bacteria, particularly rhizobacteria which are found in and around the root zone of the plant, to aid plant development and growth (Adesemoye et al., 2009; Vejan et al., 2016; Méndez-Bravo et al., 2018). Of the numerous activities that rhizobacteria employ to aid plant growth, bacterial volatile organic compounds (BVCs) have emerged as a key area of investigation over the last two decades (Ryu et al., 2003; Farag et al., 2006; Farag et al., 2017). Governments worldwide are scrambling to find solutions to the climate crisis which our planet faces both now and into the future. A major driver of this climate change is our reliance on intensive agricultural practices to produce food for humanity and particularly, where red meat is highly consumed, our livestock (Garnett 2009; Gren et al., 2019). The recent Paris climate accord has highlighted the need for our planet to prevent an average temperature increase of 2 °C in order to avoid an irreversible 'greenhouse' warming effect. However, with a projected worldwide population expected to reach ~9 billion people by 2050, how can we feed humanity in a more sustainable, affordable and environmentally friendly way? Biopesticides and their derivatives are projected to generate revenue of over $11 billion by 2022

(https://www.alliedmarketresearch.com/biopesticides-market). However, this may increase as plant growth-promoting rhizobacteria (PGPR) and their respective BVCs become an ever more attractive option for agricultural applications across the world (Heenan-Daly et al., 2019).

BVCs can act in two ways to promote plant growth; firstly, they can directly inhibit the growth of fungal and bacterial phytopathogens so as to prevent them from damaging or killing a potential host (Vespermann et al., 2007; Asari et al., 2016). Secondly, they can act to modulate gene expression within the sensor plant perceiving the volatile signal, often involving genes involved in growth, defense and nutrient acquisition (Gutiérrez-Luna et al., 2010; Farag et al., 2013; Zamioudis et al., 2015; Hernández-Calderón et al., 2018; Romera et al., 2019). This study investigates the effect of BVCs on plant growth and defense in *S. tubersoum* L. cv. 'Golden Wonder' utilising a novel co-cultivation method, and solid phase micro-extraction gas chromatography/ mass spectrometry (SPME-GC/MS). It is widely reported that during *in-vitro* experimentation the substrate on which the bacterial isolate proliferates can affect the composition of its corresponding VOC profile, having both positive and negative effects on growth (Blom et al., 2011a; Blom et al., 2011b). This can have implications for the effect of BVCs applied both in the lab and in the field with conditions such as temperature and humidity affecting the volatilisation of these compounds and thus, potentially, their respective

effect on growth (Chung et al., 2016). The effect of BVCs on modulation of gene expression related to defense, growth and nutrient acquisition has been demonstrated in numerous studies since the publications of Ryu et al. (2003) and Ryu et al. (2004) regarding the volatile-mediated regulation of gene expression in *Arabidopsis thaliana*. Indeed, this activity, rather than the direct inhibition of phytopathogens, has become a more attractive option for improved agricultural practice. BVCs can "prime" the target plant by activation of ISR (Choi et al., 2014).

The aim of this study was to investigate the effect of BVCs on plant growth with five rhizobacterial isolates obtained from Irish potato soils growing on two different media types in the presence of *S. tuberosum* L. cv. 'Golden Wonder' within a passively-ventilated growth system. Studies concerning the effect of BVCs on plant growth employ either closed or open growth systems which can affect the experimental outcome and as a consequence, can potentially misrepresent the efficiency of these molecules in any potential agricultural field application. SPME-GC/MS was used in this study to qualitatively determine the VOC profiles of bacterial isolates in three different media types, each with varying levels of carbon content. Transcriptomic analysis using MACE (massive analysis of cDNA ends) RNA-seq allowed identification of differentially expressed genes in *S. tubersousm* in response to BVCs from *Bacillus amyloliquefaciens* FZB24® and *Serratia fonticola* LAC2.

Materials and Methods:

S. tuberosum Tissue Culture Maintenance:

Microplants of *S. tuberosum* L. cv. 'Golden Wonder' were supplied by the TOPS potato centre (Department of Agricultural, Food and Marine, Raphoe, Donegal, Ireland). Stocks were maintained on ½ strength Murashige and Skoog (M+S) agar consisting of: 2.2 g basal M+S medium (Sigma Aldrich #5519), 15 g sucrose, 6 g agar (Sigma Aldrich) per litre with final pH adjusted to 5.80. Nodal sections of microplants were subcultured on fresh media under aseptic conditions every four to five weeks in Microbox® 'TP 3000' growth chambers equipped with 'XXL+' filters (Combiness, Belgium). The microplants were kept in a growth room at 23 °C under a 16 h d^{-1} photoperiod and a photosynthetic photon flux (PPF) of 225 µmol m^{-1} s^{-1}.

Bacterial Stock Maintenance:

The commercial PGPR strain *Bacillus amyloliquefaciens* FZB24® and the following isolates from Irish potato soils *Serratia fonticola* LAC2, *Pseudomonas azotoformans* BRP14, *Bacillus toyonensis* BMC10, *Bacillus mycoides* LAM7 and *Serratia myotis* LAM10 were used in all experiments. Bacterial isolates were maintained on tryptic soy agar (TSA) (Sigma Aldrich). A single bacterial colony was streaked on TSA in a 90 mm Petri dish (Sarstedt, Germany) and allowed to grow overnight at 28 °C ± 2 °C or until visible colonies were observed. Cultures were sealed using Parafilm and stored at 4 °C for four to five

weeks or maintained long term at -80 °C in a 50% glycerol solution. The 16S rRNA sequences for potato soil isolates are deposited in GenBank under the accession numbers MT373405, MT373394, MT373410, MT373402 and MT373403.

GC/MS Analysis of BVCs from Selected Rhizobacteria:

SPME-GC/MS analysis was utilised to determine VOC profiles of rhizobacteria according to Velivelli et al. (2015) with slight modifications. SPME fibres were sourced from Supelco, (Sigma Aldrich) and conditioned prior to use as per manufacturer's instructions. Rhizobacterial isolates were grown overnight on plates of TSA and incubated at 28 ± 2 °C. A single colony from each isolate was used to inoculate 20 ml glass vials (Sigma Aldrich) containing 9 ml of TSB, MR-VP or M+S under aseptic conditions. The vials were sealed with metal crimp caps (Sigma Aldrich) fitted with rubber septa (Sigma Aldrich) ensuring that they were gastight. All components were autoclaved individually prior to inoculation. Vials were incubated at 28 °C ± 2 °C for 24–72 h at 170 rpm. Volatile analysis was carried out after 24, 48, and 72 h using a Shimadzu GC/MS-QP5000 equipped with a DB-5 column (30 m × 0.25 mm × 0.25 μm). Sample vials were placed in an incubator at 50 °C. The SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane (DCP, 50/30 μm)) was then inserted and exposed to each rhizobacterial BVC sample in the vial headspace for 40 min to allow adsorption of respective volatiles. The fibre containing the volatiles was then desorbed at

210 °C for 1 min in the injection port of a gas chromatograph coupled to a mass spectrometer. The GC/MS run time was 22 min, and the injection port was operated in a split mode with a constant He flow of 1.0 ml min^{-1}. The initial oven temperature was set at 33 °C for 3 min, increased to 180 °C at a rate of 10 °C min^{-1}, further increased to 220 °C at a rate of 40 °C min^{-1}, and with a final hold for 5 min at 220 °C. Mass spectra were obtained in the electron ionization (EI) mode at 70 eV, with a continuous scan from 40 to 250 m/z. The SPME fibre was conditioned after each run for 10 min at 210 °C in the injection port and a cleaning run 'burn-off' was conducted before the next sample was analysed. Identification of the resulting VOCs was done by comparison of mass spectra, and the retention times with those of available standards and the mass spectra data of the volatile compounds were also compared with those in the NIST05/21, SZTERP mass spectra library (similarity index >90 %).

Plant/Bacteria Co-Cultivation for BVC Exposure:

Four nodal sections of four-week-old stock plants of *S. tubersoum* L. cv. 'Golden Wonder' were placed in polyurethane foams (supplied by the School of Biological, Earth and Environmental Sciences, UCC, Ireland) in magenta containers imbibed with 50 ml autotrophic medium (½ strength M+S basal medium (Sigma Aldrich #5519) 2.2 g per litre, pH adjusted to 5.80). These planted magentas were placed in 'TP5000+TPD5000' Microbox® growth chambers equipped with 'XXL+' filters (Combiness, Belgium). These growth chambers were

placed in a growth room under a 16 h photoperiod at 23 °C for two weeks to allow the microplants to acclimatise to surrounding environmental conditions. Following this the plantlets were exposed to BVCs by dropping 20 µl of each bacterial culture at OD 600nm = 1.0 on the centre of a 50 mm Petri dish containing either Methyl Red-Voges Proskeur (MR-VP) broth (Sigma Aldrich) supplemented with 14 g agar (Sigma Aldrich) per litre; or ½ strength M+S media containing 2.2 g M+S basal medium (Sigma Aldrich #5519), 15 g sucrose and 6 g agar per litre. The bacterial inoculum was allowed to air-dry under aseptic conditions for ~15 minutes and the Petri dish was then placed inside the Microbox® growth chamber containing the microplants under aseptic conditions. The microplants were exposed to the resulting rhizobacterial BVCs for a period of four weeks. Control microplants were set up with either un-inoculated axenic media or no media at all. Experiment was repeated twice with four technical replicates and each technical replicate consisted of four microplants.

RNA Extraction:

Microplants exposed to BVCs from LAC2 (L), FZB24 (F) or absolute control (C) or control with axenic media (M) were cultivated as described above in an independent experiment. Plants were harvested under aseptic conditions and RNA extractions from stem tissue were carried out using the QIAGEN® RNeasy™ plant extraction kit according to the manufacturer's specifications. To eliminate genomic DNA contamination the Ambion® TURBO™ DNAse kit was used

according to manufacturer's specifications (Life Technologies, Carlsbad, CA, USA). RNA quantification was measured using a Nanodrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were immediately snap frozen in liquid nitrogen and stored at -80°C to prevent degradation of RNA.

MACE Sequencing:

Massive Analysis of cDNA (MACE) is a 3'mRNA sequencing method based on the analysis of Illumina reads derived from fragments that originate from 3' mRNA ends (Müller et al., 2014). The samples were prepared by GenXPro GmbH (Frankfurt, Germany) using the MACE-Kit v1 according to the manual of the manufacturers (GenXPro GmbH). Briefly, RNA was fragmented and polyadenylated mRNA was enriched by poly-A specific reverse transcription. A specific adapter was ligated to the 5'ends and the 3' ends were amplified by competitive PCR. Duplicate reads as determined by the implemented unique molecular identifiers (TrueQuant IDs) were removed from the raw dataset. Low quality sequence-bases were removed by the software cutadapt (https://github.com/marcelm/cutadapt/) and poly(A)-tails were clipped by an in-house Python-Script. The RNA-seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE160297.

Statistical and Bioinformatic Analysis:

Growth data was statistically analysed using SPSS 25 (IBM). Two-way analysis of variance (ANOVA) was used to determine the interaction effect of bacterial isolate and media type on plant growth. Significant differences among BVC treatments were estimated using one-way between groups ANOVA with Tukey test for post-hoc comparisons. For transcriptomic analysis, Deseq2 version 1.24 was used on raw counts provided by GenXPro, removing genes with no counts or only a single count across all samples prior to analysis. Genes were considered to be differentially expressed if they had a Benjami Hochberg adjusted p-value of (< 0.05) and absolute log2 fold change (≥ 2). Gene Ontology (GO) enrichment was performed using GoSeq version 1.36.0 with significant GO terms identified as those with adjusted p-values (< 0.05) for over-represented terms. Gene IDs, descriptions and GO terms were retrieved from Ensembl Biomart v2.40.5 using *Solanum tuberosum* (SolTub_3.0) as the reference. The pheatmap function from the pheatmap v1.0.12 package was used to plot the heat map with clustering distance for rows and columns set to correlation, scale set to row, and row and column clustering set to use complete linkage hierarchical clustering. R version 3.6.1 was used for all analysis. The UpSetR plots were created using UpSetR version 1.4.0 using the gene IDs of genes that were differentially expressed in each comparison.

Results:

Effect of BVCs on Plant Dry Weight and Stem Length:

To test the effect of BVCs from isolates cultured on MR-VP and M+S on plant dry weight and stem length, a 50 mm Petri dish inoculated with each respective isolate, or none in the case of media control treatments, was placed in a TP5000+TPD5000 Microbox® growth chamber to allow BVCs to diffuse within the chamber thus exposing the plants within the open Magenta vessel to each of the respective isolate BVCs, the inclusion of the XXL+ grade allowed maximal passive-ventilation between the growth chamber and outer atmosphere (Fig 1).

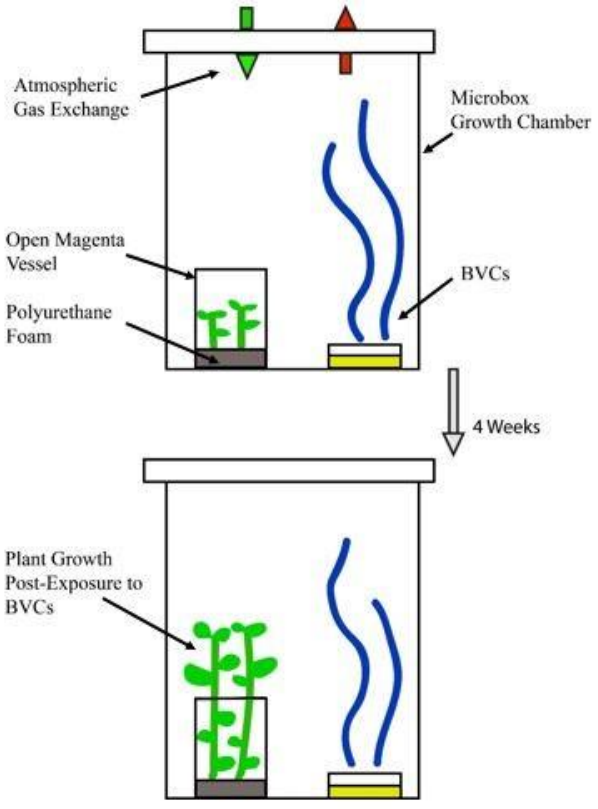


Fig 1. Plants growing in polyurethane foam imbibed with 50 ml M+S media. Bacteria release BVCs from respective growth media and plants are exposed to BVCs within the growth chamber. Gas exchange between the atmosphere and the growth chamber is mediated by the Microbox® growth chamber technology. Plants are co-cultivated with respective physically-separated bacteria for four weeks.

Two-way ANOVA was carried out to examine the relationship between bacterial isolate, media type and their interaction effect on plant growth and the resulting dry weight and stem length respectively. The interaction effect of isolate*media on dry weight was not statistically significant ($p = 0.083$) ($p < 0.01$). The interaction effect of isolate*media on stem length was also not statistically significant ($p = 0.055$) ($p < 0.01$).

To further examine the main effects of isolate and media on dry weight and stem length, one-way between groups ANOVA was carried out for isolates cultured on both types of media and their effects were determined respectively (Fig. 2). All isolate BVCs increased plant dry weight and stem length compared to plants in both controls when isolates were cultured on MR-VP. When cultured on M+S, all isolate BVCs also increased dry weight compared to both controls and five of six isolates increased stem length compared to both controls. Of the isolates cultured on MR-VP media, BRP14 and LAM7 had the greatest overall effect on biomass increase with a 2.57- and 2.69-fold increase in dry weight compared to that of the control with axenic media treatment respectively.

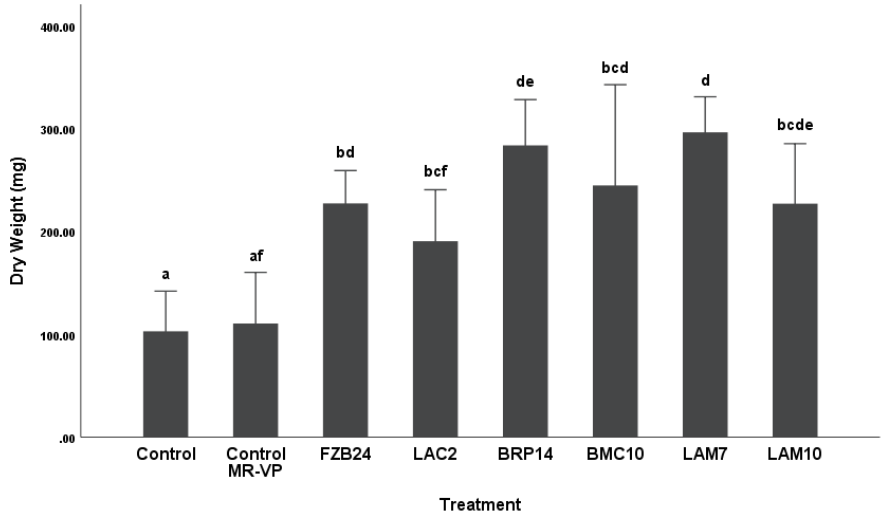


Fig 2. Growth response of plants exposed to BVCs from isolates growing on solid MR-VP media. Dry weight of plants is measured in (mg), error bars represent confidence interval of the mean 99% (n = 8). Isolates which share a common letter are not significantly different to one another ($p > 0.01$) according to one-way between groups ANOVA with post-hoc Tukey test.

No significant differences in dry weight were observed between respective *Bacillus* isolates (FZB24, BMC10, LAM7) cultured on MR-VP, additionally no significant differences were observed between the two respective *Serratia* isolates (LAC2, LAM10), however LAC2 was the only isolate significantly less effective at promoting plant growth when compared to LAM7 and BRP14 ($p > 0.01$). All isolates growing on M+S media significantly increased dry weight compared to the control treatment (Fig 3).

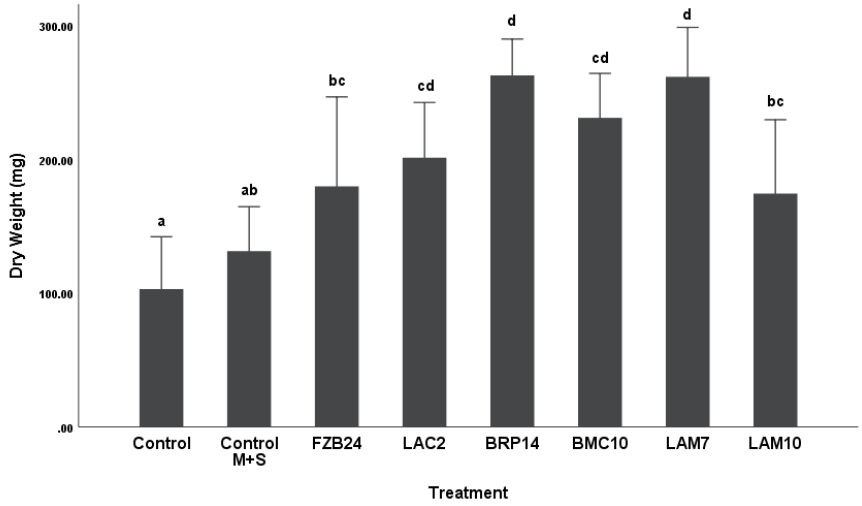


Fig 3. Growth response of plants exposed to BVCs from isolates growing on solid M+S media. Dry weight of plants is measured in (mg), error bars represent confidence interval of the mean 99% (n = 8). Isolates which share a common letter are not significantly different to one another ($p > 0.01$) according to one-way between groups ANOVA with post-hoc Tukey test.

The isolates FZB24 and LAM10 did not display a significant increase in growth compared to the control treatment with axenic M+S media ($p > 0.01$). The remaining isolates LAC2, BMC10, LAM7 and BRP14 significantly increased biomass compared to the axenic media control treatment ($p < 0.01$). The increase in biomass was again most pronounced in plants treated with BRP14 and LAM7 where dry weight was increased by ~2-fold compared to the control treatment. Differences in stem length were not as pronounced as dry weight between both media types. In plants treated with MR-VP-derived BVCs, only BRP14 and LAM7 were significantly different from both control treatments ($p < 0.01$). The *Bacillus* isolate BMC10 was also found to significantly increase stem length compared to the axenic control treatment ($p < 0.01$) (Fig. 4). No significant differences were observed between the control treatments and the FZB24, LAC2 and

LAM10 isolates ($p > 0.01$). The effect of M+S-derived BVCs on stem length was not significantly different between the test isolates and the control treatments ($p > 0.01$) (Fig. 5), indicating that media type can affect the rate of growth with respect to stem length.

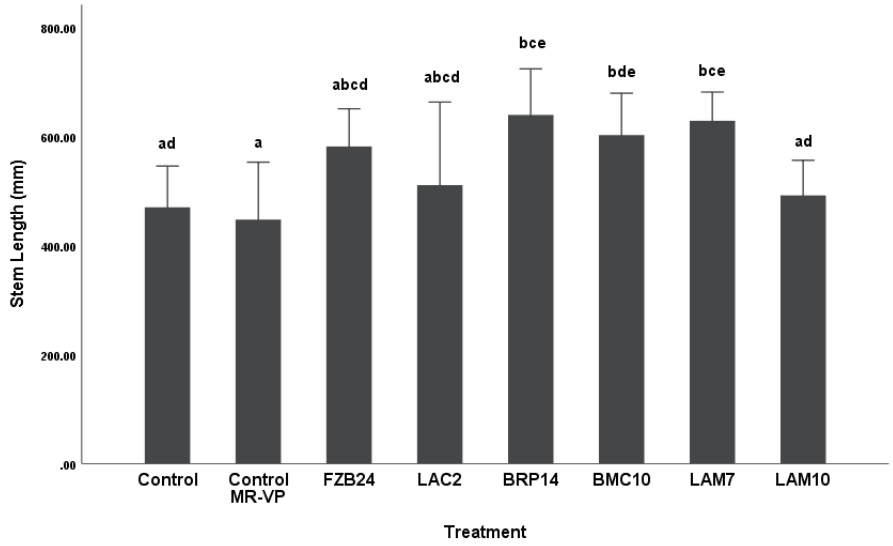


Fig 4. The effect on plant stem length of BVCs from isolates growing on solid MR-VP media. Stem length of plants is measured in (mm), error bars represent confidence interval of the mean (99%) (n = 8). Isolates which share a common letter are not significantly to one another ($p > 0.01$) according to one-way between groups ANOVA with post-hoc Tukey test.

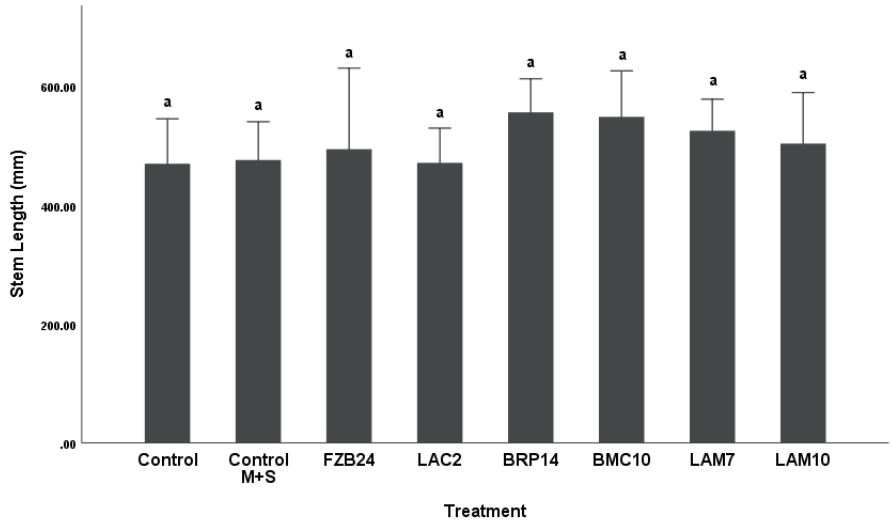


Fig 5. The effect on plant stem length of BVCs from isolates growing on solid M+S media. Stem length of plants is measured in (mm), error bars represent confidence interval of the mean (99%) (n = 8). Isolates which share a common letter are not significantly to one another ($p > 0.01$) according to one-way between groups ANOVA with post-hoc Tukey test.

Detection of BVCs from isolates cultured in three different media types:

Of the 70 BVCs detected across all isolates and media, alcohols were the most abundant (11.2%), followed by alkanes (9.1%), ketones (7%) and aldehydes (6.3%). Other chemical groups detected include the amines, alkenes, pyrazines and alkynes. All six isolates were capable of producing two BVCs, 2,5-dimethylpyrazine when cultured in TSB and hexamethylcyclotrisiloxane when cultured in M+S. In fact, all three pyrazines listed were only detected when isolates were cultured in TSB and LAC2 was the only isolate to emit each of these three pyrazines with trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine detected in addition to 2,5-dimethylpyrazine. The production of pyrazines from TSB-derived bacterial cultures has been reported previously (Rees et al., 2017).

Further analysis revealed media type influenced the number and scope of BVCs emitted by isolates. Of the three media types, M+S media was responsible for the least amount of BVCs detected, only 25 BVCs in total were detected in M+S-cultured isolates, with alkanes (24%) and ketones (32%) being the most prevalent. The best performing isolate in terms of BVC emission potential from M+S was BRP14 which emitted 11 (44%) of these 25 BVCs, in contrast, FZB24 emitted just one detectable BVC when cultured in M+S, namely hexamethylcyclotrisiloxane.

The greatest number of BVC emissions was observed when isolates were cultured in MR-VP with 40 BVCs detected in total, alcohols (30%) and alkanes (15%) were the most prevalent. The greatest amount of BVCs detected from isolates cultured in MR-VP was from LAC2 which emitted 19 (47.5%) of the 40 BVCs detected. Unlike the earlier observation in M+S, BRP14 emitted the least amount of BVCs when cultured in MR-VP with only six (15%) being detected from this isolate. A total of 33 BVCs were emitted from isolates cultured in TSB with alcohols (27.2 %) and ketones (21.2%) being the most prevalent. Similar to the observation in MR-VP, LAC2 emitted the greatest number of BVCs, 15 (45.4%) in total, while BRP14 emitted the least with eight (24.2%) BVCs detected (Table 1).

Unique BVCs were identified in a number of isolate BVC emissions, for example, LAC2 emitted nine BVCs unique to this isolate in our study, among them 2-nonanone, 2-heptadecanone and 2-undecanone which were observed in the emission profiles associated with growth in all three media types. There were 10 unique BVCs emitted by BRP14 and one of these was common to all three media types namely 1-undecene, while other BVCs include 2-nonanol, 1,4-undecadiene, nonanal decanal, 1-nonene, flouroethyne, 2-octenal, formic acid and pentanoic acid. BMC10 emitted five unique BVCs; 4-methyldecane, 2,6-dimethylundecane, ethyne, ethylene oxide and cyclobutanol, but only when cultured in TSB. LAM10 was found to emit three unique BVCs; acetaldehyde, methyl silane and 2-butynoic acid, FZB24

emitted just one unique BVC namely hydroxyurea and LAM7 emitted seven unique BVCs; 1-octanol, 1-tetradecanol, tridecanol, benzene ethanol, 2-ethynyloxy-ethanol, 1,3-bis(1,1-dimethyl-ethyl)benzene and dimethyl disulfide, the latter of which has been shown to both promote (Meldau et al., 2013) and slightly inhibit plant growth (Cordovez et al., 2018). A large amount of overlap was observed in the capability of LAC2 and LAM7 to emit the same BVCs as one another namely dimethylamine, 2-butanamine, 1-decanol, 1-dodecanol, 2-tridecanone, 2-methyl-1-butanol and 1-heptadecanol, representing 10% of all BVCs detected in our analysis.

Table 1. Volatile organic compound production from six bacterial isolates growing in tryptic soy broth (TSB), liquid MR-VP and liquid M+S.

Volatile Organic Compound	TSB	MR-VP	M+S
3-hydroxy-2-butanone	FZB24, BMC10, LAM7, LAM10	FZB24, BMC10, LAM7	BMC10
2,3-butanediol	FZB24, BMC10, LAM7, LAM10	FZB24, BMC10, LAM7	Not Detected
2,5-dimethylpyrazine	FZB24, LAC2, BRP14, BMC10, LAM7, LAM10	Not Detected	Not Detected
Hexamethylcyclotrisiloxane	FZB24, BRP14, BMC10, LAM10	FZB24, LAC2, BMC10, LAM10	BRP14, BMC10, LAC2, FZB24, LAM7, LAM10
3-methyl-1-butanol	LAC2, BRP14, LAM7	LAC2, LAM7	LAC2, LAM7
Dimethylamine	LAC2	LAC2, LAM7	Not Detected
2-butanamine	LAM7	LAC2	Not Detected
1-decanol	LAC2, LAM7	LAC2, LAM7	Not Detected
1-dodecanol	LAC2, LAM7	LAC2, LAM7	Not Detected
2-undecanone	LAC2	LAC2	LAC2
2-tridecanone	LAC2, LAM7	LAC2	LAC2, LAM7
2-heptadecanone	LAC2	LAC2	LAC2

1-methyldecylamine	LAC2	Not Detected	Not Detected
Dodecane	BMC10	FZB24, LAM7	Not Detected
2-piperidinone	Not Detected	LAC2, BMC10, LAM7, FZB24	Not Detected
2-nonanone	LAC2	LAC2	LAC2
1-undecene	BRP14	BRP14	BRP14
Benzaldehyde	FZB24, BMC10, LAM10	BMC10	Not Detected
Dimethyldisulfide	LAM7	Not Detected	Not Detected
2-methyl-1-butanol	Not Detected	LAM7	LAC2
2-ethyl-1-pentanol	Not Detected	LAC2	Not Detected
Hexadecane	BMC10	FZB24, LAC2, LAM7, LAM10	BRP14
2-ethyl-1-hexanol	BMC10, BRP14, LAM10	BMC10, FZB24	Not Detected
2-nonanol	BRP14	Not Detected	Not Detected
1,4-undecadiene	BRP14	Not Detected	Not Detected
2,9-dimethyldecane	Not Detected	BRP14, LAM10	BRP14, LAM10
Decane	Not Detected	BRP14	BMC10
1-octanol	Not Detected	LAM7	Not Detected
1-heptadecanol	LAM7	LAM7, LAC2	Not Detected
Cyclopropyl carbinol	LAC2, BMC10, LAM10	LAC2	Not Detected
1-tetradecanol	Not Detected	LAM7	Not Detected
2-propanamine	LAC2, BMC10	Not Detected	Not Detected
2-octanamine	LAM10	Not Detected	Not Detected
1-tridecanol	Not Detected	LAM7	Not Detected
Undecane	Not Detected	Not Detected	BRP14
Pentanal	Not Detected	LAC2	Not Detected
Octanal	Not Detected	LAC2	Not Detected
Nonanal	Not Detected	BRP14	Not Detected
Decanal	Not Detected	BRP14	Not Detected
3-methylbutanal	Not Detected	Not Detected	LAC2
3-tridecanone	LAC2	Not Detected	Not Detected
4-methyl-2-heptanone	LAM10	Not Detected	BRP14, BMC10
Hexadecanal	Not Detected	LAC2	Not Detected

Di-tert-butyl-dicarbonate	LAM10	Not Detected	BRP14, BMC10, LAM7
5-methylundecane	Not Detected	LAM7, LAC2	BRP14
2-ethyl-4-methyl-1-pentanol	LAC2, LAM10	Not Detected	BMC10, LAM7
Pentanoic Acid	Not Detected	BRP14	BRP14
4-methyldecane, 2,6-dimethylundecane, Ethyne, Ethylene oxide, Cyclobutanol	BMC10	Not Detected	Not Detected
1-nonene, Flouroethyne, 2-octenal	BRP14	Not Detected	Not Detected
Benzene ethanol, 2-ethynyloxy-ethanol, 1,3-bis(1,1-dimethyl-ethyl)benzene	Not Detected	LAM7	Not Detected
Formic acid	Not Detected	Not Detected	BRP14
4-methyldecane	Not Detected	LAM10	BRP14
Trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine	LAC2	Not Detected	Not Detected
Hydroxyurea	Not Detected	FZB24	Not Detected
1,3-dioxolane	Not Detected	BMC10, LAM10	LAM7
4-methyl-2-pentanone, 2-ethoxy-2-methylpropane	Not Detected	Not Detected	BMC10, LAM7
4,6-dimethyl-2-heptanone	Not Detected	Not Detected	LAM7
Acetaldehyde	Not Detected	LAM10	Not Detected
Methyl Silane	Not Detected	LAM10	Not Detected
2-butynoic acid	Not Detected	Not Detected	LAM10

Transcriptomic response to BVC exposure:

A principal component analysis revealed that there were distinct differences in gene expression between BVC treatment and control groups, with treatment accounting for 80% of the variance in gene expression between groups in the first two principal components (Fig 6.)

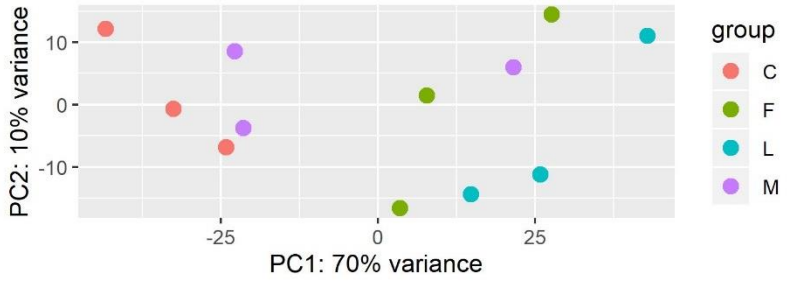


Fig 6. Plot of the first two principal components of a PCA generated from the expression data (count data with variance stabilising transformation from DeSeq2) coloured by condition showing that samples in each condition group together. (C) represents the control group, (M) represents the axenic media control group, (F) represents the FZB 24 BVC treatment group and (L) represents the LAC2 BVC treatment group.

A heatmap of differentially expressed genes (DEGs) between control and BVC treatments demonstrates that for the most part the respective treatments co-cluster with one another except for one of the MR-VP axenic media control treatments (M2) (Fig 7).

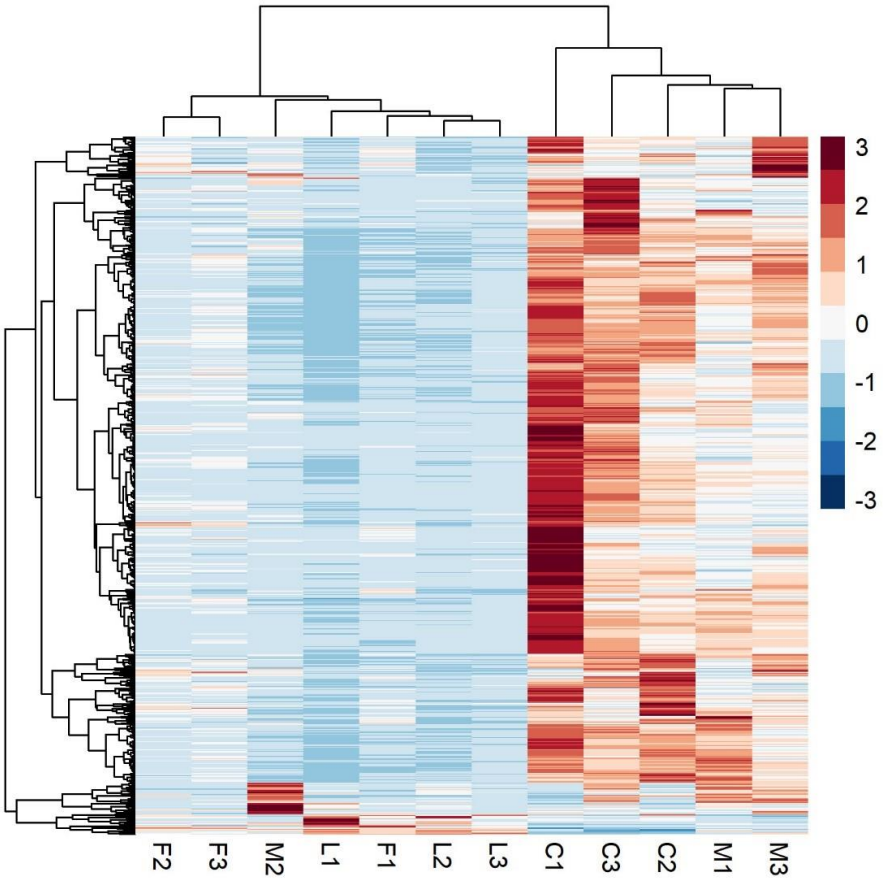


Fig 7. A heatmap of RNA-Seq expression z-scores computed for genes that are significantly differentially expressed Benjamini-Hochberg adjusted p-value ($p < 0.05$) in any comparison. The genes (rows) and samples (columns) are clustered using the Pearson Correlation distance and complete linkage hierarchical clustering. The colour code shows the row z-score, with a red colour indicating higher expression of a gene and blue colour indicating lower expression of a gene. This heatmap demonstrates that C and M groups and L and F groups cluster together and that the genes involved have higher expression in the C and M groups compared with the L and F groups. (C) represents the control group, (M) represents the axenic media control group, (F) represents the FZB 24 BVC treatment group and (L) represents the LAC2 BVC treatment group.

Overall, BVC treatment within our experimental set up, results in the relative downregulation of genes compared to the control treatments, regardless of whether the respective BVC-emitting isolate is from the *Bacillus* or *Serratia* genus. Enrichment of three gene ontologies was observed following plant exposure to BVCs emitted by LAC2, all of which were associated with photosynthesis. Two belong to the

biological process, GO:0019684 (photosynthesis, light reaction) and GO:0009772 (photosynthetic electron transport in photosystem II) while the third belonged to molecular function GO:0045156 (electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity).

MACE RNA-seq revealed that in all, 52 genes were upregulated between treatments and, the greatest number of DEGs, 17 altogether, was observed in the (C vs M) comparison. The LAC2 BVC-treated plants had the next highest levels of upregulated genes with 14 and 13 DEGs observed in the (L vs C) and (L vs M) comparisons respectively. Very little upregulation of gene expression was observed in FZB24 BVC-treated plants with the (F vs C), (F vs M) and (F vs L) yielding two, three and three DEGs respectively (Fig. 8).

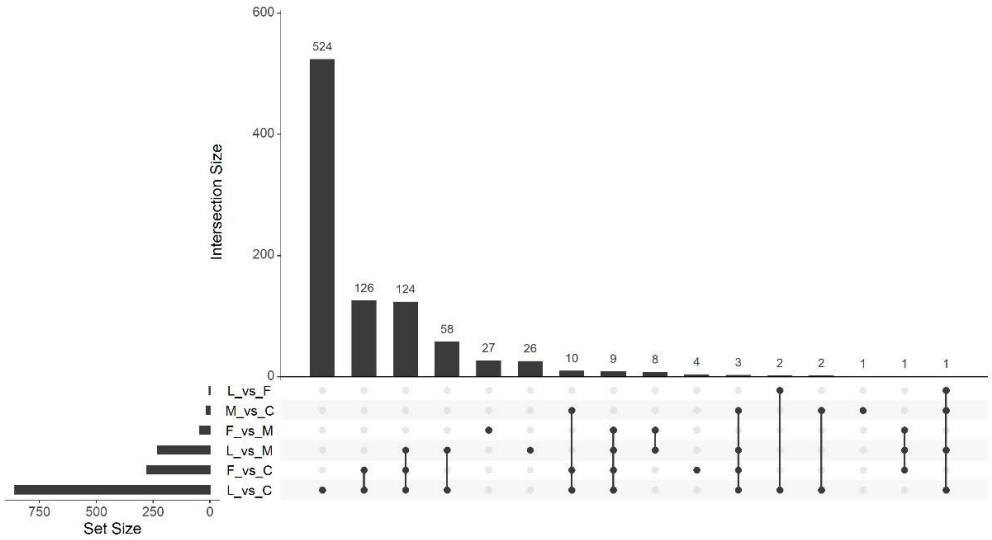


Fig 8. UpSetR plot showing numbers of downregulated differentially expressed genes (DEGs), ($p < 0.05$) and Log2 fold change (≥ 2.0). Any DEGs common to plant treatments are shown by connected dots. Cumulative numbers of DEGs are calculated by adding numbers associated with dots in each respective treatment row. (C) represents the control group, (M) represents the axenic media control group, (F) represents the FZB 24 BVC treatment group and (L) represents the LAC2 BVC treatment group.

Table 2. Numbers of upregulated and downregulated genes between BVC treatments from isolates cultured on MR-VP media according to MACE RNA-seq.

Treatment Comparison	Upregulated Genes	Downregulated Genes
FZB24 vs Control	2	277
LAC2 vs Control	14	859
Control vs Axenic MR-VP	17	2
FZB24 vs LAC2	3	0
FZB24 vs Axenic MR-VP	3	45
LAC2 vs Axenic MR-VP	13	230
Total	**52**	**1,413**

Table 3. Upregulated genes from PGSC genome annotations in response to BVC exposure

Treatment	Gene ID	Gene Name	Log2FC	Reference
LAC2 vs Control	PGSC0003DMG400007123	Phytosulfokine peptide	2.075	Igarashi et al., 2012
	PGSC0003DMG400008309	Chlorophyll a/b binding protein	2.097	Zhu et al., 2020
	PGSC0003DMG400022062	Aspartic proteinase	2.413	Resjö et al., 2019
	PGSC0003DMG400028333	Plastid division	2.125	Chen et al., 2018

		regulator MinE		
	PGSC0003DMG400026831	Conserved gene of unknown function	4.159	N/A
	PGSC0003DMG400020916	Fibrillarin homolog	4.499	Rajamaki et al., 2020
	PGSC0003DMG401031741	UPA16	2.274	Kay et al., 2009
	PGSC0003DMG400005792	Nucleoside diphosphate kinase	3.760	Lin et al., 2015
	PGSC0003DMG402025318	Zinc finger (CCCH-type) protein	2.075	Bogamuwa and Jang 2014
	PGSC0003DMG400033693	UPA16	3.046	Kay et al., 2009
	PGSC0003DMG400008713	Hsp20/alpha crystallin family protein	3.918	Zhao et al., 2018
LAC2 vs Axenic MR-VP	PGSC0003DMG400000493	Carbonic anhydrase	2.074	DiMario et al., 2018
	PGSC0003DMG400008309	Chlorophyll a/b binding protein	2.289	Zhu et al., 2020
	PGSC0003DMG400005884	Conserved gene of unknown function	3.288	Lekota et al., 2019
	PGSC0003DMG400013416	Chlorophyll a-b binding protein 3C, chloroplastic	2.074	Zhu et al., 2020

	PGSC0003DMG400004301	Chlorophyll a,b binding protein type I	2.185	Zhu et al., 2020
	PGSC0003DMG400007796	DNA-directed RNA polymerase II largest subunit	2.663	Zhou et al., 2017
	PGSC0003DMG400033046	Dof zinc finger protein	2.161	Venkatesh and Park 2015
	PGSC0003DMG400013412	Chlorophyll a-b binding protein 3C	2.455	Gao and Bradeen 2016
	PGSC0003DMG400012838	Non-specific lipid-transfer protein	4.402	N/A
	PGSC0003DMG400012839	Non-specific lipid-transfer protein	4.392	N/A
FZB24 vs Axenic MR-VP	PGSC0003DMG400030593	Proteinase inhibitor IIa	2.088	Rehman et al., 2017

Of the upregulated genes observed in the (L vs C) comparison a number of genes associated with photosynthetic processes and cell division were observed such as a chrolophyll a/b binding protein and the plastid division regulator MinE along with genes associated with a possible stress and/or defence responses such as Zinc finger (CCCH-type) protein and Hsp20/alpha crystallin family protein. The (L vs M)

comparison also resulted in the upregulation of genes associated with photosynthesis where four chlorophyll a/b binding proteins were identified. In the (F vs M) comparison the gene for protease inhibitor IIa was the only identifiable transcript from the PGSC genome (Table 3).

MACE RNA-seq revealed that 1,413 genes were downregulated across all comparisons, the greatest difference being observed in (L vs C) with 859 DEGs identified (Table 2). As in the upregulation response, BVC treatment from the LAC2 isolate was responsible for the highest amount of DEG downregulation. The lowest amount of downregulated DEGs was in the (F vs L) comparison with no DEGs observed (Fig. 9). Generally, downregulation was far more pronounced than upregulation in BVC-treated plants with a large proportion of the identifiable DEGs being related to defence and stress responses (Table 4).

Table 4. Downregulated genes from PGSC genome annotations directly involved in or related to plant defense and stress responses identified post-BVC exposure

Treatment	Gene ID	Gene Name	Log2FC	Reference
FZB24 vs Control	PGSC0003DMG400033029	Enhanced disease susceptibility 1 protein	-2.412	El Oirdi and Bouarab 2007; Venugopal et al., 2009
	PGSC0003DMG400007742	NBS-coding resistance gene protein	-2.190	N/A

	PGSC0003DMG400025437	Stress responsive gene 6 protein, Srg6	-2.043	Zhuang et el., 2012
	PGSC0003DMG400000241	Spotted leaf protein	-2.178	Zeng et al., 2004
	PGSC0003DMG400020587	Rpi-vnt1	-3.014	Roman et al., 2017
	PGSC0003DMG400029220	Tospovirus resistance protein A	-2.464	de Oliveira et al., 2018
	PGSC0003DMG400004413	Tm-2 protein	-2.414	Mayann et al., 2018
	PGSC0003DMG400012025	UPF0497 membrane protein 8	-2.714	Resjö et al., 2019
	PGSC0003DMG400002362	GYF domain-containing protein	-2.321	Wu et al., 2017
LAC2 vs Control	PGSC0003DMG402030235	Disease resistance protein R3a	-2.570	Engelhardt et al., 2012
	PGSC0003DMG402029058	Disease resistance protein	-3.213	N/A
	PGSC0003DMG400012025	UPF0497 membrane protein 8	-4.407	Resjö et al., 2019
	PGSC0003DMG400021733	TNP2	-2.359	He at al., 2000
	PGSC0003DMG400030239	CC-NBS-LRR protein	-3.895	Goyer et al., 2015
	PGSC0003DMG400017065	Disease resistance protein RGA1	-2.679	Huang et al., 2005
	PGSC0003DMG400030462	Avr9/Cf-9 rapidly elicited protein 216	-3.260	Rowland et al., 2005
	PGSC0003DMG400033029	Enhanced disease susceptibility 1 protein	-3.932	El Oirdi and Bouarab 2007; Venugopal

			et al., 2009
PGSC0003DMG400020099	RPM1 interacting protein 4 transcript 2	-2.052	Lee et al., 2015
PGSC0003DMG401031196	WRKY transcription factor 16	-2.213	Phukan et al., 2016
PGSC0003DMG400001550	TSI-1 protein	-2.9046	Dahal et al., 2009
PGSC0003DMG400028904	SGT1	-2.260	Yu et al., 2019
PGSC0003DMG400007742	NBS-coding resistance gene protein	-3.812	N/A
PGSC0003DMG400032555	NAC domain protein	-2.174	Kwenda et al., 2016
PGSC0003DMG400004053	Protein CASP	-2.641	N/A
PGSC0003DMG400021331	PEN1	-2.231	Collins et al., 2003; Nielsen et al., 2012
PGSC0003DMG400016769	Double WRKY type transfactor (StWRKY8)	-2.163	Gao et al., 2020
PGSC0003DMG400009238	Leucine-rich repeat family protein	-2.109	N/A
PGSC0003DMG402029995	TMV resistance protein N	-3.358	Qian et al., 2018
PGSC0003DMG400031839	RING-H2 finger protein ATL2	-2.898	Serrano et al., 2004
PGSC0003DMG400009634	Bacterial spot disease resistance protein 4	-3.018	Schornack et al., 2004
PGSC0003DMG400025437	Stress responsive gene 6 protein, Srg6	-2.531	Malatrasi et al., 2002
PGSC0003DMG400024365	Rpi-vnt1	-4.841	Roman et al., 2017
PGSC0003DMG400019104	Heat shock protein 70 (HSP70)-interacting protein	-2.261	Jiang et al., 2014

	PGSC0003DMG400000241	Spotted leaf protein	-2.903	Zeng et al., 2004
	PGSC0003DMG400044116	Beta-glucan-binding protein 4	-2.599	Fliegman et al., 2004
	PGSC0003DMG400004327	Leucine-rich repeat resistance protein	-2.123	N/A
	PGSC0003DMG404026432	Tir-nbs-lrr resistance protein	-4.939	Li et al., 2017
	PGSC0003DMG401011899	Tospovirus resistance protein B	-2.391	de Oliveira et al., 2018
	PGSC0003DMG400029220	Tospovirus resistance protein A	-3.154	de Oliveira et al., 2016
	PGSC0003DMG400019667	Potato resistance I2GA-SH23-3	-2.709	Huang et al., 2005
	PGSC0003DMG401030700	Resistance gene	-3.319	Gu et al., 2020
	PGSC0003DMG400029371	DNA-binding protein NtWRKY3	-2.209	Liu et al., 2004
	PGSC0003DMG400047046	Nematode resistance	-2.675	Goyer et al., 2015
	PGSC0003DMG400047346	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1	-2.153	Bentham et al., 2020
	PGSC0003DMG402026149	Mitogen-activated protein kinase kinase kinase	-2.646	Pitzschke et al. 2009
LAC2 vs Axenic MR-VP	PGSC0003DMG400030462	Avr9/Cf-9 rapidly elicited protein 216	-2.871	Rowland et al., 2005
	PGSC0003DMG400020099	RPM1 interacting protein 4 transcript 2	-2.547	Lee et al., 2015
	PGSC0003DMG400033029	Enhanced disease susceptibility 1 protein	-2.758	Venugopal et al., 2009

	PGSC0003DMG400019232	GRAS2	-2.851	Chang et al., 2015
	PGSC0003DMG400019824	JA-induced WRKY protein	-2.030	Wiesel et al., 2015
	PGSC0003DMG401031196	WRKY transcription factor 16	-2.644	Phukan et al., 2016
	PGSC0003DMG400028904	SGT1	-2.472	Yu et al., 2019
	PGSC0003DMG400007742	NBS-coding resistance gene protein	-2.564	N/A
	PGSC0003DMG400021331	PEN1	-2.248	Collins et al., 2003; Nielsen et al., 2012
	PGSC0003DMG401030700	Resistance gene	-2.550	Gu et al., 2020
	PGSC0003DMG400002562	F-box/kelch-repeat protein	-2.366	Wang et al., 2017
FZB24 vs Axenic MR-VP	PGSC0003DMG400002562	F-box/kelch-repeat protein	-2.417	Wang et al., 2017

Discussion:

For almost two decades the interactions of BVCs with plants has been the focus of intense research. These interactions can be directly observed in the sensor plant, usually through the induction of a defence response via the upregulation of pathogenesis-related (PR) genes which are key for induced systemic resistance (ISR) or through an increased rate in plant growth and accumulation of biomass (Ryu et al., 2004; Kim et al., 2015). BVCs can also have indirect effects on plants via the

inhibition of bacterial and especially fungal phytopathogens (Giorgio et al., 2015) and have also been observed to increase the rate of micronutrient-uptake in sensor plants, leading to a potentially biofortified harvest in crop plants (Orozco-Mosqueda et al., 2013).

We devised a novel growth system in which to study the effects of BVCs on potato plants in an enclosed, passively-ventilated experimental setup. The model plant *Arabidopsis thaliana* is often the predominant target in lab-based in vitro studies of the BVC-mediated effects on plants (Bee Park et al., 2013; Sharifi and Ryu 2016; Bhattacharyya et al., 2016). Here, we present the growth effects of BVCs on potato to study their effects on a major staple food crop plant. The type of growth system, either open or closed, in which to observe the effect of microbial VOCs on plants, phytopathogens and animals has been widely researched and discussed in recent years (Kai et al., 2007; Popova et al., 2014; Park et al. 2015; Kai et al., 2016; Sharifi and Ryu 2018; Song et al., 2019). Extrapolating how these respective systems affect volatile concentrations, mixtures of volatiles, responses of test organisms and the combined spatiotemporal effects of these parameters has made the definitive translation of effects from the lab setting to the field more difficult. The physical nature of VOCs, such as a high vapour pressure, low boiling point and low molecular weight (< 300 Da) lends to their short-term proximity to target organisms in the field, although a number of methodologies have been developed to mitigate this effect (Song and Ryu 2013; Fincheira et al., 2019; Fincheira et al., 2020). In the lab, closed systems have been the popular

choice for observing the experimental effects of VOCs on target organisms due to their relative ease-of-use and affordability. The most common approach is the use of split-plate Petri dishes, often referred to as 'I plates', where VOCs can diffuse from one chamber of the plate to the other over a central dividing septum (Velivelli et al., 2015). Closed systems have a number of drawbacks however, the first being that the build-up and subsequent increased concentration of VOCs within these systems is highly unlikely to occur in an open system or especially in the field. The second is that given the right conditions, the effect of VOCs can be detrimental, or even lethal, to the target organism (Blom et al., 2011b), as we observed in plants that were exposed to BVCs from TSB-cultured isolates. Open systems represent a more true-to-life effect of VOCs on target organisms (Kai and Piechulla, 2009) but have the drawback of laborious mechanical apparatus such as pumps and the added expense which these can bring. Our approach was to find a "middle ground", where the outer and inner atmosphere of the system could passively interact and exchange between one another to avoid an excessive accumulation of bacterial and indeed plant-derived volatiles, while still maintaining an axenic environment within the system thanks to the Microbox® growth chamber, along with providing relative ease-of-use and affordability. Polyurethane (PE) foam was selected in which to grow potato microplants as it represented a more neutral substrate in which to cultivate the plant compared to soil, which has been used in some closed systems (Park et al., 2015; Tahir et al., 2017). Indeed, while

sterilised soil may be lacking live microbes with potential PGP activity, it will still retain organic matter which can as a nutrient source thus masking or inadvertently misrepresenting, the absolute effect of BVCs on PGP potential, this is an implication which deserves further study. The additional advantage of using PE foam is that, like soil, the foam is a porous matrix thus allowing potential diffusion of BVCs to the root system. However, in our study root proliferation throughout the foam made it too difficult to obtain an adequate root length and root dry weight as it was not possible to simply wash away or melt the foam, as can be achieved using soil or agar, from the roots.

This study identified VOCs from six bacterial isolates from three genera; *Bacillus*, *Serratia* and *Pseudomonas* cultured in three different types of liquid media via SPME-GC/MS. The growth medium of the bacteria is known to alter the blend of BVCs which are emitted (Blom et al., 2011a) and taking this into account we selected TSB, MR-VP and M+S liquid media from which to assess both the BVC-producing capability and variety from selected isolates. We identified 70 different BVCs in total, some of which are widely reported in the literature such as 2,3-butanediol, 3-hydroxy-2-butanone, 2,5-dimethylpyrazine, 2-nonanone, 2-tridecanone and 3-methyl-1-butanol (Ryu et al., 2003; Velivelli et al., 2015; Asari et al., 2016; Fincheira and Quiroz 2018; Fincheira and Quiroz 2019). Some BVCs were also observed to be isolate-specific such as 1-undecene, 2-nonanone and 1-tetradecanol produced by BRP14, LAC2 and LAM7 respectively. Interestingly, dimethyl disulphide, widely reported as a PGP volatile among many

PGP bacterial isolates (Meldau et al., 2013; Tyc et al., 2017) was only detected once in our analysis by a single strain, LAM7, and only when cultured in TSB.

When isolates cultured on MR-VP agar were co-cultivated with potato microplants there was a significant increase in growth between the control and all BVC treatments. This may be attributed to the carbon-rich composition of this media and the subsequent blend and/or concentration of BVCs present within the growth system. The most common BVCs observed between isolates utilising MR-VP as a carbon source were hexamethylcyclotrisiloxane, hexadecane and 2-piperidinone, with four of six isolates emitting these particular BVCs, the latter was recently described as an insecticidal constituent from a biocontrol fertiliser (Qiu et al., 2019). The next most common were 3-hydroxy-2-butanone and 2,3-butanediol identified in the headspace of three isolates, FZB24, BMC10 and LAM7 all of which are members of *Bacillus*. The largest increases in dry biomass were observed after exposure to BVCs from LAM7 and BRP14, 2.69- and 2.57-fold greater than that of the axenic media control respectively, suggesting that the volatile blends from these isolates cultured in MR-VP are strong growth-promoters. Our analysis identified 2,3-butanediol and 3-hydroxy-2-butanone within the headspace of *B. mycoides* (LAM7), interestingly these were not detected in the headspace of *B. mycoides* strains (CHT2401) and (CHT2402) in a previous study (Huang et al., 2018). This may indicate that bacterial strains within a particular species may harbour the ability to employ strain-specific BVCs for

exploitation of particular ecological niches under the correct environmental conditions. Stem length was also significantly increased after exposure to BVCs from both LAM7 and BRP14 in comparison to both control treatments. There was a total of 18 volatiles identified in the LAM7/MR-VP BVC blend and consisted of, amongst others, 3-methyl-1-butanol, 1-decanol, 1-dodecanol, hexadecane, 1-octanol and 1-tridecanol which were also identified as growth promoters in previous studies (Camarena-Pozos et al., 2019; Velivelli et al., 2015; Bee Park et al., 2013; Velázquez-Becerra et al., 2010; Pérez-Flores et al., 2017). In BRP 14/MR-VP the predominant BVC, from a total blend of just five, was identified as 1-undecene, a particularly potent volatile in the biocontrol effect against some fungal phytopathogens mediated by many *Pseudomonas* strains (Kong et al., 2020). The greatest effect on stem length was observed with plants exposed to BVCs of BRP14/MR-VP.

A similar trend was seen in dry weight measurements from M+S-derived BVC treatments as was observed in MR-VP, however LAC2 BVCs marginally increased dry weight compared to those of FZB24 when cultured on M+S. No significant differences in stem length were observed post-exposure to M+S-cultured isolates to that of the respective control treatments. These differences in growth observed between both media types can be explained by the composition of the respective media, with MR-VP being a more carbon-rich media than M+S. Indeed, we observed that proliferation of isolates across the media surface was far more apparent in isolates which were cultured

on MR-VP, while isolates cultured on M+S rarely expanded their radial growth beyond the point at which their starting culture was dropped on to the media. Members of the genus *Serratia* are reported as BVC-emitting biocontrol and PGP agents (Wenke et al., 2019) and while their BVC-mediated inhibition of phytopathogenic growth has been well reported, the effect of volatiles from two *Serratia* strains in this study, LAC2 and LAM10, were often not as effective as those of *Bacillus* and *Pseudomonas* at increasing plant growth. LAC2 was the only isolate observed to produce 2-undecanone, 2-heptadecanone and 2-nonanone which have been the subject of particular research focus in recent years, particularly 2-nonanone both in the preservation of commercially important fruits such as strawberry from *Botrytis cinerea* (Abarca et al., 2017), as an inhibitor of bacterial quorum sensing, a disruptor of protein folding in bacteria and inducer of plant defence (Chernin et al., 2011; Melkina et al., 2017; Báez-Vallejo et al., 2020).

For transcriptomic analysis of potato plants exposed to BVCs in our system the isolates LAC2 and the commercially available isolate FZB24, members of *Serratia* and *Bacillus* respectively were chosen as the BVC source due to the fact they only share three common BVCs when grown on MR-VP, namely hexamethylcyclotrisiloxane, 2-piperidinone and hexadecane. Additionally, species of these genus have been well documented to emit antimicrobial and growth-promoting BVCs (Fincheira et al., 2016; Tahir et al., 2017). Therefore, we expected to see noticeable alterations in the plant transcriptomic landscape in response to these isolates. Interestingly, only 52 genes

were upregulated across treatments, whereas 1,413 were downregulated. In BVC-treated plants, LAC2 was responsible for the upregulation of 12 genes (Fig. 9) with some involved in photosynthetic activity, such as a number of genes coding for chlorophyll a/b binding proteins and the plastid division regulator MinE. Genes associated with defence and stress responses were also upregulated such as phytosulfokine peptide, aspartic proteinase and zinc finger (CCCH-type) protein. FZB24 was responsible for the upregulation of just one identifiable gene from the PGSC potato genome, proteinase inhibitor IIa, which is known to be involved in the defence response against phytopathogens and insect pests (Rehman et al., 2017) (Table 3). The gene phytosulfokine peptide has been reported as an attenuator of pattern-triggered immunity in *Arabidopsis* and acts as a positive regulator of growth by balancing resource allocation between defence and growth (Igarshi et al., 2012).

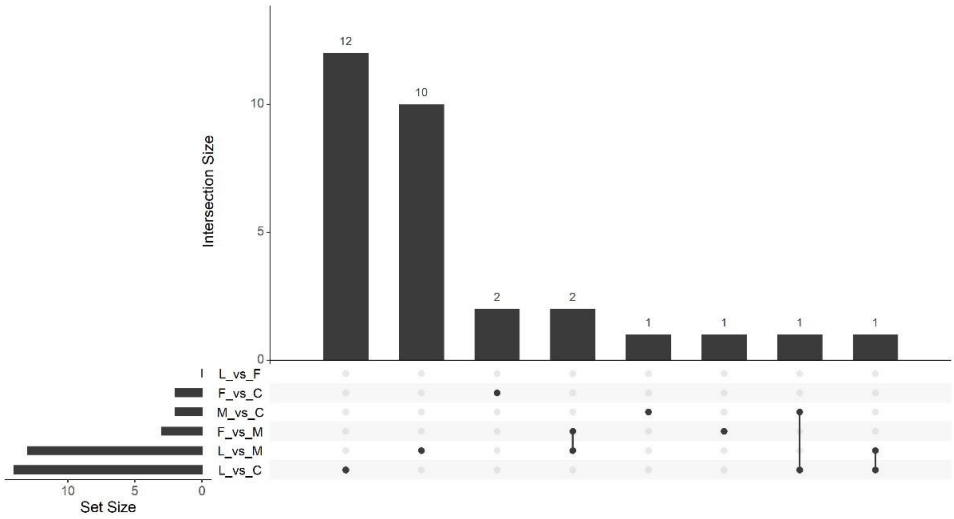


Fig 9. UpSetR plot showing numbers of upregulated differentially expressed genes (DEGs), ($p < 0.05$) and Log2 fold change (≥ 2.0). Any DEGs common to plant treatments are shown by connected dots. Cumulative numbers of DEGs are calculated by adding numbers associated with dots in each respective treatment row. (C) represents the control group, (M) represents the axenic media control group, (F) represents the FZB 24 BVC treatment group and (L) represents the LAC2 BVC treatment group.

Unexpectedly, many of the downregulated genes were related to defence and stress responses. A similar response has been reported previously from the pure PGP volatile 1-decene emitted by the beneficial fungus *Trichoderma* which downregulated stress and defence genes such as WRKY transcription factors (Lee et al., 2019). Three WRKY transcriptions factors were also downregulated in our study. Five DEGs related to stress and/or defence were commonly downregulated in (L vs C) and (F vs C) namely enhanced disease susceptibility 1 protein (EDS1); stress responsive gene 6 protein, Srg6; Rpi-vnt1; tospovirus resistance protein A and UPF0497 membrane protein 8. The protein (EDS1) has been shown, in association with salicylic acid (SA), to mediate redundant functions in R protein-mediated defence signalling confirmed by simultaneous mutations of EDS1 and a salicylic acid-synthesising enzyme named SID2 resulting in a compromised defence and/or hypersensitive response mediated by R proteins which contain a coiled-coil domain residing in their N-terminal domains (Venugopal et al., 2009). A membrane protein involved in cell wall strengthening, UPF0497, was shown to be upregulated in the PTI response to *Phytophthora infestans* (Resjö et al., 2019), this class of membrane protein was also downregulated in our study. The fact that there are only three BVCs shared in common between LAC2 and FZB24 suggests that these BVCs may be responsible for the induction of the five commonly downregulated DEGs between these isolates and is worthy of future investigation.

Three DEGs downregulated in our analysis code for proteins named PEN1, EDS1 and SGT1 which are known to play crucial roles in plant defence, especially non-host resistance (NHR) to fungal pathogens. These three genes work in a hierarchical manner and sometimes directly co-operate to mediate the hosts innate defence response. PEN1 (PENETRATION 1) is involved in diminishing the successful entry of phytopathogens into the plant cell. *PEN1* encodes a syntaxin anchored in the plasma membrane which is part of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex and is required for the timely arrival of papillae which contain callose and extracellular membrane material that aids penetration resistance (Collins et al., 2003; Nielsen et al., 2012). As part of the SNARE complex, the PEN1 protein forms part of the activation of pre-invasion defence in the resistance to powdery mildews in *Arabidopsis* (Lipka et al., 2008). In the event that the frontline defence of the PEN1/SNARE complex fails, mechanisms to deal with post-invasion defence come into play, where EDS1 and SGT1 play a significant role. The post-invasion NHR response requires EDS1, pyhtoalexin-deficient 4 (PAD4) and senescence-associated gene 101 (SAG101) and has been confirmed through mutational analyses (Lipka et al., 2005; Stein et al., 2006). These proteins are homologous to lipases and comprise a regulatory node that is crucial for basal defence against the oomycete *Hyaloperonospora parasitica*, SA-mediated signalling and some resistance pathways mediated by R-genes (Kwon et al., 2008). As part of a crucial NHR response EDS1 is necessary for the resistance,

including the hypersensitive response, mediated by TIR (Toll/interleukin 1 receptor)-containing proteins (Hu et al., 2005; Wiermer et al., 2005). SGT1 is also essential for the correct functioning of several R genes and the post-invasion NHR response in Arabidopsis and is a highly conserved constituent required for the function of certain Skp1/Cullin/F-Box protein (SCF)-type E3 ubiquitin ligase complexes (Austin et al., 2002; Liu et al., 2004; Holt et al., 2005; Azevedo et al., 2006; Yu et al., 2019). While EDS1 and SGT1 have been shown to be essential components of the plant innate immune system, the never-ending arms race between pathogen and host can turn these genes into clandestine pathogenic weapons. El Oirdi and Bouarab (2007) demonstrated that *B. cinerea* can essentially flank the defences of the Solanaeceous member *Nicotiana benthamiana* using a molecular distraction. EDS1 and SGT1 are part of the control mechanism for SA-dependent disease resistance towards biotrophic pathogens, while resistance against necrotrophic pathogens is mediated through the jasmonic acid (JA) pathway (McDowell and Dangl. 2000; da Cunha et al., 2006). The SA and JA pathways are known to be antagonistic (Mur et al., 2006). EDS1 acts as an activator of SA signalling but as a repressor of JA/Ethylene signalling (Brodersen et al., 2006), therefore the authors concluded that the activation of EDS1 and SGT1 signalling dampened the JA signalling response thus allowing *B. cinerea* pathogenesis to progress through exploitation of the plants own innate immune system (El Oirdi and Bouarab, 2007).

The use of this passively-ventilated system has proved viable for observing the effect of BVCs on plant growth, and may mitigate the effects observed in closed growth systems which often lack sufficient space for many plants other than *Arabidopsis thaliana* seedlings to grow freely. In many in vitro BVC-plant studies, fold changes in plant growth promotion can be quite striking (Blom et al., 2011a; Park et al., 2015). In our study the growth promotion is more modest, perhaps owing to the passive ventilation of the system, which may more closely mimic BVC kinetics found in open natural environments. The observation that genes involved in attenuating and mediating the plant immune response, both upregulated and downregulated respectively, demonstrates that BVC-mediated communication with the plant can involve more than just the upregulation of defence genes. Rather, the induction of a careful "dance" between up- and downregulation of a suite of genes, perhaps to optimise the pre-colonisation internal environment of the host for an endophytic symbiont. If the bacterial symbiont has an epiphytic life strategy in the rhizosphere of the host plant and depends on host-derived root exudates as a carbon source, this dance may maintain the overall health status of the host by dampening the negative physiological effects of an over-reactive defence response. The use of this system is quite affordable and may allow for the study of more cumbersome or older plants that cannot be studied in relatively restrictive I-plates. Future analysis on the effect of plant type, plant substrate, microbe, microbial growth media, filter porosity and other parameters relevant to BVC-plant interactions in this

system may help to elucidate specific genes or pathways activated in response to a particular single BVC or blend. The opportunity also presents itself to analyse the tripartite effect of fungal/bacterial/plant volatile organic compound interactions within this experimental setup. Systems such as this can help elucidate genes and/or their pathways modulated via BVC exposure for a better understanding of their effect on plant health and ultimately for better solutions to the looming concern of global food security in an ecologically uncertain future.

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References:

Abarca, R. L., Rodríguez, F. J., Guarda, A., Galotto, M. J., Bruna, J. E., Perez, M. A. F.,...& Padula, M. (2017). Application of β-cyclodextrin/2-nonanone inclusion complex as active agent to design of antimicrobial packaging films for control of *Botrytis cinerea*. *Food and Bioprocess Technology*, *10*(9), 1585-1594.

Adesemoye, A. O., Torbert, H. A., & Kloepper, J. W. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microbial ecology*, *58*(4), 921-929.

Asari, S., Matzén, S., Petersen, M. A., Bejai, S., & Meijer, J. (2016). Multiple effects of *Bacillus amyloliquefaciens* volatile compounds: plant growth promotion and growth inhibition of phytopathogens. *FEMS Microbiology Ecology*, *92*(6), fiw070.

Austin, M. J., Muskett, P., Kahn, K., Feys, B. J., Jones, J. D., & Parker, J. E. (2002). Regulatory role of SGT1 in early R gene-mediated plant defenses. *Science*, *295*(5562), 2077-2080.

Azevedo, C., Betsuyaku, S., Peart, J., Takahashi, A., Noel, L., Sadanandom, A.,...& Shirasu, K. (2006). Role of SGT1 in resistance protein accumulation in plant immunity. *The EMBO Journal*, *25*(9), 2007-2016.

Báez-Vallejo, N., Camarena-Pozos, D. A., Monribot-Villanueva, J. L., Ramírez-Vázquez, M., Carrión-Villarnovo, G. L., Guerrero-Analco, J. A.,...& Reverchon, F. (2020). Forest tree associated bacteria for potential biological control of *Fusarium solani* and of *Fusarium kuroshium*, causal agent of *Fusarium* dieback. *Microbiological Research*, *235*, 126440.

Bee Park, H., Lee, B., Kloepper, J. W., & Ryu, C. M. (2013). One shot-two pathogens blocked: exposure of *Arabidopsis* to hexadecane, a long chain volatile organic compound, confers induced resistance against both *Pectobacterium carotovorum* and *Pseudomonas syringae*. *Plant signaling & behavior*, *8*(7), e24619.

Bentham, A. R., De la Concepcion, J. C., Mukhi, N., Zdrzałek, R., Draeger, M., Gorenkin, D., ... & Banfield, M. J. (2020). A molecular roadmap to the plant immune system. *Journal of Biological Chemistry*, *295*(44), 14916-14935.

Bhattacharyya, D., Yu, S. M., & Lee, Y. H. (2015). Volatile compounds from *Alcaligenes faecalis* JBCS1294 confer salt tolerance in *Arabidopsis thaliana* through the auxin and gibberellin pathways and differential modulation of gene expression in root and shoot tissues. *Plant growth regulation*, *75*(1), 297-306.

Bloemberg, G. V., & Lugtenberg, B. J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current opinion in plant biology*, *4*(4), 343-350.

Blom, D., Fabbri, C., Connor, E. C., Schiestl, F. P., Klauser, D. R., Boller, T.,..& Weisskopf, L. (2011a). Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. *Environmental microbiology*, *13*(11), 3047-3058.

Blom, D., Fabbri, C., Eberl, L., & Weisskopf, L. (2011b). Volatile-mediated killing of *Arabidopsis thaliana* by bacteria is mainly due to hydrogen cyanide. *Applied and environmental microbiology*, *77*(3), 1000-1008.

Bogamuwa, S. P., & Jang, J. C. (2014). Tandem CCCH zinc finger proteins in plant growth, development and stress response. *Plant and Cell Physiology*, *55*(8), 1367-1375.

Brodersen, P., Petersen, M., Bjørn Nielsen, H., Zhu, S., Newman, M. A., Shokat, K. M.,...& Mundy, J. (2006). *Arabidopsis* MAP kinase 4 regulates salicylic acid-and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *The Plant Journal*, *47*(4), 532-546.

Camarena-Pozos, D. A., Flores-Núñez, V. M., López, M. G., López-Bucio, J., & Partida-Martínez, L. P. (2019). Smells from the desert: Microbial volatiles that affect plant growth and development of native and non-native plant species. *Plant, cell & environment*, *42*(4), 1368-1380.

Chabot, R., Beauchamp, C. J., Kloepper, J. W., & Antoun, H. (1998). Effect of phosphorus on root colonization and growth promotion of maize by bioluminescent mutants of phosphate-solubilizing *Rhizobium leguminosarum* biovar phaseoli. *Soil Biology and Biochemistry*, *30*(12), 1615-1618.

Chang, Y. H., Yan, H. Z., & Liou, R. F. (2015). A novel elicitor protein from *Phytophthora parasitica* induces plant basal immunity and systemic acquired resistance. *Molecular plant pathology*, *16*(2), 123-136.

Chen, C., MacCready, J. S., Ducat, D. C., & Osteryoung, K. W. (2018). The molecular machinery of chloroplast division. *Plant physiology*, *176*(1), 138-151.

Chernin, L., Toklikishvili, N., Ovadis, M., Kim, S., Ben-Ari, J., Khmel, I., & Vainstein, A. (2011). Quorum-sensing quenching by rhizobacterial volatiles. *Environmental microbiology reports*, *3*(6), 698-704.

Chung, J. H., Song, G. C., & Ryu, C. M. (2016). Sweet scents from good bacteria: case studies on bacterial volatile compounds for plant growth and immunity. *Plant molecular biology*, *90*(6), 677-687.

Choi, H. K., Song, G. C., Yi, H. S., & Ryu, C. M. (2014). Field evaluation of the bacterial volatile derivative 3-pentanol in priming for induced resistance in pepper. *Journal of chemical ecology*, *40*(8), 882-892.

Collins, N. C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J. L.,...& Schulze-Lefert, P. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. *Nature*, *425*(6961), 973-977.

Cordovez, V., Schop, S., Hordijk, K., de Boulois, H. D., Coppens, F., Hanssen, I.,...& Carrion, V. J. (2018). Priming of Plant Growth Promotion by Volatiles of Root-Associated *Microbacterium* spp. *Applied and environmental microbiology*, *84*(22).

Dahal, D., Heintz, D., Van Dorsselaer, A., Braun, H. P., & Wydra, K. (2009). Pathogenesis and stress related, as well as metabolic proteins are regulated in tomato stems infected with *Ralstonia solanacearum*. *Plant Physiology and Biochemistry*, *47*(9), 838-846.

da Cunha, L., McFall, A. J., & Mackey, D. (2006). Innate immunity in plants: a continuum of layered defenses. *Microbes and infection*, *8*(5), 1372-1381.

de Oliveira, A. S., Boiteux, L. S., Kormelink, R., & Resende, R. O. (2018). The Sw-5 gene cluster: tomato breeding and research toward orthotospovirus disease control. *Frontiers in plant science*, *9*, 1055.

De Oliveira, A. S., Koolhaas, I., Boiteux, L. S., Caldararu, O. F., Petrescu, A. J., Oliveira Resende, R., & Kormelink, R. (2016). Cell death triggering and effector recognition by Sw-5 SD-CNL proteins from resistant and susceptible tomato isolines to Tomato spotted wilt virus. *Molecular plant pathology*, *17*(9), 1442-1454.

del Carmen Orozco-Mosqueda, M., Macías-Rodríguez, L. I., Santoyo, G., Farías-Rodríguez, R., & Valencia-Cantero, E. (2013). *Medicago truncatula* increases its iron-uptake mechanisms in response to volatile organic compounds produced by *Sinorhizobium meliloti*. *Folia microbiologica*, *58*(6), 579-585.

DiMario, R. J., Machingura, M. C., Waldrop, G. L., & Moroney, J. V. (2018). The many types of carbonic anhydrases in photosynthetic organisms. *Plant science*, *268*, 11-17.

Edgar, R., Domrachev, M., & Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research*, *30*(1), 207-210.

Engelhardt, S., Boevink, P. C., Armstrong, M. R., Ramos, M. B., Hein, I., & Birch, P. R. (2012). Relocalization of late blight resistance protein R3a to endosomal compartments is associated with effector recognition and required for the immune response. *The Plant Cell*, *24*(12), 5142-5158.

El Oirdi, M., & Bouarab, K. (2007). Plant signalling components EDS1 and SGT1 enhance disease caused by the necrotrophic pathogen *Botrytis cinerea*. *New Phytologist*, *175*(1), 131-139.

Farag, M. A., Ryu, C. M., Sumner, L. W., & Paré, P. W. (2006). GC–MS SPME profiling of rhizobacterial volatiles reveals prospective

inducers of growth promotion and induced systemic resistance in plants. *Phytochemistry*, *67*(20), 2262-2268.

Farag, M. A., Zhang, H., & Ryu, C. M. (2013). Dynamic chemical communication between plants and bacteria through airborne signals: induced resistance by bacterial volatiles. *Journal of chemical ecology*, *39*(7), 1007-1018.

Farag, M. A., Song, G. C., Park, Y. S., Audrain, B., Lee, S., Ghigo, J. M.,...& Ryu, C. M. (2017). Biological and chemical strategies for exploring inter-and intra-kingdom communication mediated via bacterial volatile signals. *nature protocols*, *12*(7), 1359.

Fincheira, P., Parra, L., Mutis, A., Parada, M., & Quiroz, A. (2017). Volatiles emitted by *Bacillus* sp. BCT9 act as growth modulating agents on *Lactuca sativa* seedlings. *Microbiological Research*, *203*, 47-56.

Fincheira, P., Rubilar, O., Espinoza, J., Aniñir, W., Méndez, L., Seabra, A. B., & Quiroz, A. (2019). Formulation of a controlled-release delivery carrier for volatile organic compounds using multilayer O/W emulsions to plant growth. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *580*, 123738.

Fincheira, P., & Quiroz, A. (2018). Microbial volatiles as plant growth inducers. *Microbiological research*, *208*, 63-75.

Fincheira, P., Quiroz, A., Medina, C., Tortella, G., Hermosilla, E., Diez, M. C., & Rubilar, O. (2020). Plant growth induction by volatile organic compound released from solid lipid nanoparticles and nanostructured lipid carriers. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 124739.

Fincheira, P., & Quiroz, A. (2019). Physiological response of Lactuca sativa exposed to 2-nonanone emitted by *Bacillus* sp. BCT9. *Microbiological research*, *219*, 49-55.

Fincheira, P., Venthur, H., Mutis, A., Parada, M., & Quiroz, A. (2016). Growth promotion of *Lactuca sativa* in response to volatile organic compounds emitted from diverse bacterial species. *Microbiological research*, *193*, 39-47.

Flaishman, M. A., Eyal, Z., Zilberstein, A., Voisard, C., & Haas, D. (1996). Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. *MPMI-Molecular Plant Microbe Interactions*, *9*(7), 642-645.

Fliegmann, J., Mithöfer, A., Wanner, G., & Ebel, J. (2004). An ancient enzyme domain hidden in the putative β-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. *Journal of Biological Chemistry*, *279*(2), 1132-1140.

Gao, L., & Bradeen, J. M. (2016). Contrasting potato foliage and tuber defense mechanisms against the late blight pathogen *Phytophthora infestans*. *PloS one*, *11*(7), e0159969.

Gao, Y. F., Liu, J. K., Yang, F. M., Zhang, G. Y., Wang, D., Zhang, L., ... & Yao, Y. A. (2020). The WRKY transcription factor WRKY8 promotes resistance to pathogen infection and mediates drought and salt stress tolerance in *Solanum lycopersicum*. *Physiologia plantarum*, *168*(1), 98-117.

Garnett, T. (2009). Livestock-related greenhouse gas emissions: impacts and options for policy makers. *Environmental science & policy*, *12*(4), 491-503.

Giorgio, A., De Stradis, A., Lo Cantore, P., & Iacobellis, N. S. (2015). Biocide effects of volatile organic compounds produced by potential biocontrol rhizobacteria on *Sclerotinia sclerotiorum*. *Frontiers in microbiology*, *6*, 1056.

Goyer, A., Hamlin, L., Crosslin, J. M., Buchanan, A., & Chang, J. H. (2015). RNA-Seq analysis of resistant and susceptible potato varieties during the early stages of potato virus Y infection. *BMC genomics*, *16*(1), 472.

Gren, M., Moberg, E., Säll, S., & Röös, E. (2019). Design of a climate tax on food consumption: examples of tomatoes and beef in Sweden. *Journal of Cleaner Production*, *211*, 1576-1585.

Gutiérrez-Luna, F. M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., de la Cruz, H. R., & Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. *Symbiosis*, *51*(1), 75-83.

He, Z. H., Dong, H. T., Dong, J. X., Li, D. B., & Ronald, P. C. (2000). The rice Rim2 transcript accumulates in response to *Magnaporthe grisea* and its predicted protein product shares similarity with TNP2-like proteins encoded by CACTA transposons. *Molecular and General Genetics MGG*, *264*(1-2), 2-10.

Heenan-Daly, D., Velivelli, S. L., & Prestwich, B. D. (2019). The Role of Rhizobacterial Volatile Organic Compounds in a Second Green Revolution—The Story so Far. In *Field Crops: Sustainable Management by PGPR* (pp. 191-220). Springer, Cham.

Hernández-Calderón, E., Aviles-Garcia, M. E., Castulo-Rubio, D. Y., Macías-Rodríguez, L., Ramírez, V. M., Santoyo, G.,...& Valencia-Cantero, E. (2018). Volatile compounds from beneficial or pathogenic bacteria differentially regulate root exudation, transcription of iron transporters, and defense signaling pathways in Sorghum bicolor. *Plant molecular biology*, *96*(3), 291-304.

Holt, B. F., Belkhadir, Y., & Dangl, J. L. (2005). Antagonistic control of disease resistance protein stability in the plant immune system. *Science*, *309*(5736), 929-932.

Hu, G., DeHart, A. K., Li, Y., Ustach, C., Handley, V., Navarre, R.,...& Baker, B. (2005). EDS1 in tomato is required for resistance mediated by TIR-class R genes and the receptor-like R gene Ve. *The Plant Journal*, *42*(3), 376-391.

Huang, J. S., Peng, Y. H., Chung, K. R., & Huang, J. W. (2018). Suppressive efficacy of volatile compounds produced by *Bacillus mycoides* on damping-off pathogens of cabbage seedlings. *The Journal of Agricultural Science*, *156*(6), 795-809.

Huang, S., Van Der Vossen, E. A., Kuang, H., Vleeshouwers, V. G., Zhang, N., Borm, T. J.,...& Visser, R. G. (2005). Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. *The Plant Journal*, *42*(2), 251-261.

Jiang, S., Lu, Y., Li, K., Lin, L., Zheng, H., Yan, F., & Chen, J. (2014). Heat shock protein 70 is necessary for R ice stripe virus infection in plants. *Molecular Plant Pathology*, *15*(9), 907-917.

Kai, M., Effmert, U., Berg, G., & Piechulla, B. (2007). Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Archives of microbiology*, *187*(5), 351-360.

Kai, M., Effmert, U., & Piechulla, B. (2016). Bacterial-plant-interactions: approaches to unravel the biological function of bacterial volatiles in the rhizosphere. *Frontiers in microbiology*, *7*, 108.

Kai, M., & Piechulla, B. (2009). Plant growth promotion due to rhizobacterial volatiles–An effect of CO2?. *FEBS letters*, *583*(21), 3473-3477.

Kay, S., Hahn, S., Marois, E., Wieduwild, R., & Bonas, U. (2009). Detailed analysis of the DNA recognition motifs of the *Xanthomonas* type III effectors AvrBs3 and AvrBs3Δrep16. *The Plant Journal*, *59*(6), 859-871.

Kim, J. S., Lee, J., Lee, C. H., Woo, S. Y., Kang, H., Seo, S. G., & Kim, S. H. (2015). Activation of pathogenesis-related genes by the rhizobacterium, *Bacillus* sp. JS, which induces systemic resistance in tobacco plants. *The Plant Pathology Journal*, *31*(2), 195.

Kong, W. L., Li, P. S., Wu, X. Q., Wu, T. Y., & Sun, X. R. (2020). Forest tree associated bacterial diffusible and volatile organic compounds against various phytopathogenic fungi. *Microorganisms*, *8*(4), 590.

Kwenda, S., Motlolometsi, T. V., Birch, P. R., & Moleleki, L. N. (2016). RNA-seq profiling reveals defense responses in a tolerant potato cultivar to stem infection by *Pectobacterium carotovorum* ssp. *brasiliense*. *Frontiers in plant Science*, *7*, 1905.

Kwon, C., Neu, C., Pajonk, S., Yun, H. S., Lipka, U., Humphry, M.,...& El Kasmi, F. (2008). Co-option of a default secretory pathway for plant immune responses. *Nature*, *451*(7180), 835-840.

Lee, S., Behringer, G., Hung, R., & Bennett, J. (2019). Effects of fungal volatile organic compounds on *Arabidopsis thaliana* growth and gene expression. *Fungal Ecology*, *37*, 1-9.

Lee, D., Bourdais, G., Yu, G., Robatzek, S., & Coaker, G. (2015). Phosphorylation of the plant immune regulator RPM1-INTERACTING PROTEIN4 enhances plant plasma membrane H+-ATPase activity and inhibits flagellin-triggered immune responses in *Arabidopsis*. *The Plant Cell*, *27*(7), 2042-2056.

Lekota, M., Muzhinji, N., & Van der Waals, J. E. (2019). Identification of differentially expressed genes in tolerant and susceptible potato cultivars in response to *Spongospora subterranea* f. sp. *subterranea* tuber infection. *Plant Pathology*, *68*(6), 1196-1206.

Li, Y., Hu, X., Chen, J., Wang, W., Xiong, X., & He, C. (2017). Integrated mRNA and microRNA transcriptome analysis reveals miRNA regulation in response to PVA in potato. *Scientific reports*, *7*(1), 1-16.

Lin, T., Lashbrook, C. C., Cho, S. K., Butler, N. M., Sharma, P., Muppirala, U.,...& Hannapel, D. J. (2015). Transcriptional analysis of phloem-associated cells of potato. *BMC genomics*, *16*(1), 665.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M.,...& Llorente, F. (2005). Pre-and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. *Science*, *310*(5751), 1180-1183.

Lipka, U., Fuchs, R., & Lipka, V. (2008). *Arabidopsis* non-host resistance to powdery mildews. *Current opinion in plant biology*, *11*(4), 404-411.

Liu, Y., Burch-Smith, T., Schiff, M., Feng, S., & Dinesh-Kumar, S. P. (2004). Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. *Journal of Biological Chemistry*, *279*(3), 2101-2108.

Liu, Y., Schiff, M., & Dinesh-Kumar, S. P. (2004). Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. *The Plant Journal*, *38*(5), 800-809.

Maayan, Y., Pandaranayaka, E. P., Srivastava, D. A., Lapidot, M., Levin, I., Dombrovsky, A., & Harel, A. (2018). Using genomic analysis to identify tomato Tm-2 resistance-breaking mutations and their underlying evolutionary path in a new and emerging tobamovirus. *Archives of virology*, *163*(7), 1863-1875.

Malatrasi, M., Close, T. J., & Marmiroli, N. (2002). Identification and mapping of a putative stress response regulator gene in barley. *Plant molecular biology*, *50*(1), 141-150.

McDowell, J. M., & Dangl, J. L. (2000). Signal transduction in the plant immune response. *Trends in biochemical sciences*, *25*(2), 79-82.

Meldau, D. G., Meldau, S., Hoang, L. H., Underberg, S., Wünsche, H., & Baldwin, I. T. (2013). Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition. *The Plant Cell*, *25*(7), 2731-2747.

Melkina, O. E., Khmel, I. A., Plyuta, V. A., Koksharova, O. A., & Zavilgelsky, G. B. (2017). Ketones 2-heptanone, 2-nonanone, and 2-undecanone inhibit DnaK-dependent refolding of heat-inactivated bacterial luciferases in *Escherichia coli* cells lacking small chaperon IbpB. *Applied microbiology and biotechnology*, *101*(14), 5765-5771.

Méndez-Bravo, A., Cortazar-Murillo, E. M., Guevara-Avendaño, E., Ceballos-Luna, O., Rodríguez-Haas, B., Kiel-Martínez, A. L.,...& Reverchon, F. (2018). Plant growth-promoting rhizobacteria associated with avocado display antagonistic activity against *Phytophthora cinnamomi* through volatile emissions. *PLoS One*, *13*(3), e0194665.

Müller, S., Rycak, L., Afonso-Grunz, F., Winter, P., Zawada, A. M., Damrath, E.,...& Rotter, B. (2014). APADB: a database for alternative polyadenylation and microRNA regulation events. *Database*, *2014*.

Mur, L. A., Kenton, P., Atzorn, R., Miersch, O., & Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant physiology*, *140*(1), 249-262.

Nielsen, M. E., Feechan, A., Böhlenius, H., Ueda, T., & Thordal-Christensen, H. (2012). *Arabidopsis* ARF-GTP exchange factor, GNOM, mediates transport required for innate immunity and focal accumulation of syntaxin PEN1. *Proceedings of the National Academy of Sciences*, *109*(28), 11443-11448.

Park, Y. S., Dutta, S., Ann, M., Raaijmakers, J. M., & Park, K. (2015). Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochemical and Biophysical Research Communications*, *461*(2), 361-365.

Phukan, U. J., Jeena, G. S., & Shukla, R. K. (2016). WRKY transcription factors: molecular regulation and stress responses in plants. *Frontiers in plant science*, *7*, 760.

Pitzschke, A., Schikora, A., & Hirt, H. (2009). MAPK cascade signalling networks in plant defence. *Current opinion in plant biology*, *12*(4), 421-426.

Popova, A. A., Koksharova, O. A., Lipasova, V. A., Zaitseva, J. V., Katkova-Zhukotskaya, O. A., Eremina, S. I., ... & Khmel, I. A. (2014). Inhibitory and toxic effects of volatiles emitted by strains of *Pseudomonas* and *Serratia* on growth and survival of selected microorganisms, *Caenorhabditis elegans*, and *Drosophila melanogaster*. *BioMed Research International*, *2014*.

Pérez-Flores, P., Valencia-Cantero, E., Altamirano-Hernández, J., Pelagio-Flores, R., López-Bucio, J., García-Juárez, P., & Macías-Rodríguez, L. (2017). *Bacillus methylotrophicus* M4-96 isolated from maize (*Zea mays*) rhizoplane increases growth and auxin content in *Arabidopsis thaliana* via emission of volatiles. *Protoplasma*, *254*(6), 2201-2213.

Qian, L., Zhao, J., Du, Y., Zhao, X., Han, M., & Liu, Y. (2018). Hsp90 Interacts With Tm-22 and Is Essential for Tm-22-Mediated Resistance to Tobacco mosaic virus. *Frontiers in plant science*, *9*, 411.

Qiu, L., Li, J. J., Li, Z., & Wang, J. J. (2019). Production and characterization of biocontrol fertilizer from brewer's spent grain via solid-state fermentation. *Scientific reports*, *9*(1), 1-9.

Rajamäki, M. L., Sikorskaite-Gudziuniene, S., Sarmah, N., Varjosalo, M., & Valkonen, J. P. (2020). Nuclear proteome of virus-infected and healthy potato leaves. *BMC plant biology*, *20*(1), 1-16.

Rees, C. A., Nordick, K. V., Franchina, F. A., Lewis, A. E., Hirsch, E. B., & Hill, J. E. (2017). Volatile metabolic diversity of *Klebsiella pneumoniae* in nutrient-replete conditions. *Metabolomics*, *13*(2), 18.

Rehman, S., Aziz, E., Akhtar, W., Ilyas, M., & Mahmood, T. (2017). Structural and functional characteristics of plant proteinase inhibitor-II (PI-II) family. *Biotechnology letters*, *39*(5), 647-666.

Resjö, S., Zahid, M. A., Burra, D. D., Lenman, M., Levander, F., & Andreasson, E. (2019). Proteomics of PTI and Two ETI Immune Reactions in Potato Leaves. *International journal of molecular sciences*, *20*(19), 4726.

Rowland, O., Ludwig, A. A., Merrick, C. J., Baillieul, F., Tracy, F. E., Durrant, W. E.,...& Jones, J. D. (2005). Functional analysis of Avr9/Cf-9 rapidly elicited genes identifies a protein kinase, ACIK1, that is essential for full Cf-9–dependent disease resistance in tomato. *The Plant Cell*, *17*(1), 295-310.

Roman, M. L., Izarra, M., Lindqvist-Kreuze, H., Rivera, C., Gamboa, S., Tovar, J. C.,...& Ghislain, M. (2017). R/Avr gene expression study of Rpi-vnt1. 1 transgenic potato resistant to the *Phytophthora infestans* clonal lineage EC-1. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *131*(2), 259-268.

Romera, F. J., García, M. J., Lucena, C., Martínez-Medina, A., Aparicio, M. A., Ramos, J.,...& Pérez-Vicente, R. (2019). Induced

systemic resistance (ISR) and Fe deficiency responses in dicot plants. *Frontiers in Plant Science*, *10*, 287.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Wei, H. X., Paré, P. W., & Kloepper, J. W. (2003). Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *100*(8), 4927-4932.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., & Paré, P. W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant physiology*, *134*(3), 1017-1026.

Schornack, S., Ballvora, A., Gürlebeck, D., Peart, J., Ganal, M., Baker, B.,...& Lahaye, T. (2004). The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. *The Plant Journal*, *37*(1), 46-60.

Serrano, M., & Guzmán, P. (2004). Isolation and gene expression analysis of *Arabidopsis thaliana* mutants with constitutive expression of ATL2, an early elicitor-response RING-H2 zinc-finger gene. *Genetics*, *167*(2), 919-929.

Sharifi, R., & Ryu, C. M. (2016). Making healthier or killing enemies? Bacterial volatile-elicited plant immunity plays major role upon protection of *Arabidopsis* than the direct pathogen inhibition. *Communicative & integrative biology*, *9*(4), 196.

Sharifi, R., & Ryu, C. M. (2018). Revisiting bacterial volatile-mediated plant growth promotion: lessons from the past and objectives for the future. *Annals of botany*, *122*(3), 349-358.

Song, G. C., Riu, M., & Ryu, C. M. (2019). Beyond the two compartments Petri-dish: optimising growth promotion and induced resistance in cucumber exposed to gaseous bacterial volatiles in a miniature greenhouse system. *Plant methods*, *15*(1), 9.

Song, G. C., & Ryu, C. M. (2013). Two volatile organic compounds trigger plant self-defense against a bacterial pathogen and a sucking insect in cucumber under open field conditions. *International journal of molecular sciences*, *14*(5), 9803-9819.

Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B. H., Molina, A., Schulze-Lefert, P.,...& Somerville, S. (2006). *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *The Plant Cell*, *18*(3), 731-746.

Tahir, H. A. S., Gu, Q., Wu, H., Raza, W., Safdar, A., Huang, Z.,...& Gao, X. (2017). Effect of volatile compounds produced by *Ralstonia solanacearum* on plant growth promoting and systemic resistance inducing potential of *Bacillus* volatiles. *BMC plant biology*, *17*(1), 1-16.

Tyc, O., Song, C., Dickschat, J. S., Vos, M., & Garbeva, P. (2017). The ecological role of volatile and soluble secondary metabolites produced by soil bacteria. *Trends in microbiology*, *25*(4), 280-292.

Venkatesh, J., & Park, S. W. (2015). Genome-wide analysis and expression profiling of DNA-binding with one zinc finger (Dof) transcription factor family in potato. *Plant Physiology and Biochemistry*, *94*, 73-85.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Nasrulhaq Boyce, A. (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules*, *21*(5), 573.

Velivelli, S. L., Kromann, P., Lojan, P., Rojas, M., Franco, J., Suarez, J. P., & Prestwich, B. D. (2015). Identification of mVOCs from Andean rhizobacteria and field evaluation of bacterial and mycorrhizal inoculants on growth of potato in its center of origin. *Microbial ecology*, *69*(3), 652-667.

Venugopal, S. C., Jeong, R. D., Mandal, M. K., Zhu, S., Chandra-Shekara, A. C., Xia, Y.,...& Kachroo, P. (2009). Enhanced disease susceptibility 1 and salicylic acid act redundantly to regulate resistance gene-mediated signaling. *PLoS Genet*, *5*(7), e1000545.

Vespermann, A., Kai, M., & Piechulla, B. (2007). Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. *Applied and environmental microbiology*, *73*(17), 5639-5641.

Wang, J., Yao, W., Wang, L., Ma, F., Tong, W., Wang, C.,...& Xu, Y. (2017). Overexpression of VpEIFP1, a novel F-box/Kelch-repeat protein from wild Chinese Vitis pseudoreticulata, confers higher tolerance to powdery mildew by inducing thioredoxin z proteolysis. *Plant Science*, *263*, 142-155.

Wenke, K., Kopka, J., Schwachtje, J., van Dongen, J. T., & Piechulla, B. (2019). Volatiles of rhizobacteria *Serratia* and *Stenotrophomonas* alter growth and metabolite composition of *Arabidopsis thaliana*. *Plant Biology*, *21*, 109-119.

Wiermer, M., Feys, B. J., & Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Current opinion in plant biology*, *8*(4), 383-389.

Wiesel, L., Davis, J. L., Milne, L., Fernandez, V. R., Herold, M. B., Williams, J. M.,...& Birch, P. R. (2015). A transcriptional reference map of defence hormone responses in potato. *Scientific reports*, *5*, 15229.

Wu, Z., Huang, S., Zhang, X., Wu, D., Xia, S., & Li, X. (2017). Regulation of plant immune receptor accumulation through translational repression by a glycine-tyrosine-phenylalanine (GYF) domain protein. *Elife*, *6*, e23684.

Yu, G., Xian, L., Xue, H., Yu, W., Rufian, J., Sang, Y.,...& Macho, A. P. (2019). A bacterial effector protein prevents MAPK-mediated

phosphorylation of SGT1 to suppress plant immunity. bioRxiv, 641241. *Preprint at*, *10*, 641241.

Zamioudis, C., Korteland, J., Van Pelt, J. A., van Hamersveld, M., Dombrowski, N., Bai, Y.,...& Pieterse, C. M. (2015). Rhizobacterial volatiles and photosynthesis-related signals coordinate MYB 72 expression in *Arabidopsis* roots during onset of induced systemic resistance and iron-deficiency responses. *The Plant Journal*, *84*(2), 309-322.

Zeng, L. R., Qu, S., Bordeos, A., Yang, C., Baraoidan, M., Yan, H.,...& Wang, G. L. (2004). Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *The Plant Cell*, *16*(10), 2795-2808.

Zhao, P., Wang, D., Wang, R., Kong, N., Zhang, C., Yang, C., ...& Chen, Q. (2018). Genome-wide analysis of the potato Hsp20 gene family: identification, genomic organization and expression profiles in response to heat stress. *Bmc Genomics*, *19*(1), 61.

Zhou, X., Zha, M., Huang, J., Li, L., Imran, M., & Zhang, C. (2017). StMYB44 negatively regulates phosphate transport by suppressing expression of PHOSPHATE1 in potato. *Journal of experimental botany*, *68*(5), 1265-1281.

Zhuang, X., McPhee, K. E., Coram, T. E., Peever, T. L., & Chilvers, M. I. (2012). Rapid transcriptome characterization and parsing of sequences in a non-model host-pathogen interaction; pea-*Sclerotinia sclerotiorum*. *BMC genomics*, *13*(1), 668.

Zhu, T., Li, L., & Feng, L. (2020). StABI5 Involved in the Regulation of Chloroplast Development and Photosynthesis in Potato. *International journal of molecular sciences*, *21*(3), 1068.

Chapter 5

Volatile Organic Compounds from Plant Growth-Promoting Rhizobacteria enhance *Agrobacterium tumefaciens*-mediated genetic transformation in *Solanum tuberosum*

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Abstract:

Current challenges facing the world's agronomic sector are leading to new avenues in the way in which we develop and cultivate our most important crops. Volatile organic compounds (VOCs) produced by bacteria can modulate gene expression in many, if not all plant species, often resulting in the ability to better absorb micronutrients from the environment, increase photosynthesis and ward off potential phytopathogenic attack. Here we investigate the potential of plant pre-treatment with bacterial VOCs (BVCs) to improve *Agrobacterium tumefaciens*-mediated transformation (AMT) in *Solanum tuberosum* L. cv. 'Golden Wonder'. Two plant growth-promoting bacterial isolates, *Serratia fonticola* LAC2 and *Bacillus amyloliquifaciens* FZB24, were selected as BVC-emitters for co-cultivation within a Microbox® plant growth system. *S. fonticola* LAC2 BVC pre-treatment of plants was

found to consistently improve transient transformation rates compared with *B. amyloliquifaciens* FZB24 and often significantly improved transient transformation rates compared to controls. The pre-treatment of plants with BVCs prior to AMT may aid in the increased recovery of transgenic crop plants with agronomically important traits better able to feed the world's population and at the same time mitigate environmental damage from intensive agriculture.

Introduction:

In the last two decades the microbial volatilome has become a significant area of research interest under the umbrella of plant-microbe interactions (Morath et al. 2012; Heenan-Daly et al. 2019). Numerous studies have shown that BVCs promote growth through modulation of gene expression involved in defence (Ryu et al. 2004), nutrient acquisition (Orozco-Mosqueda et al. 2013; Wang et al. 2017) and hormone production (Jiang et al. 2019) or indeed through direct growth inhibition of potential pathogens (Vlassi et al. 2020). These BVCs show promise for agricultural applications and this is especially true within the European Union as there is now more stringent restrictions on the number of available plant protection products and the respective amounts with which they can be applied to commercial crops (Vlassi et al. 2020).

This poses a number of difficult-to-navigate scenarios as worldwide populations continue to grow and demand for food increases along with

societal expectations of less intensive food production practices (Garnett 2014), coupled with this, the change in climate predicted for much of Europe will allow more optimal conditions for the growth of fungal pathogens and present a greater risk of mycotoxins entering the food chain (Moretti et al. 2019). Therefore, taking advantage of plant growth-promoting rhizobacteria (PGPR) and/or their respective BVCs can help maintain Europe's food security in a restrictive agrichemical market. In the global context potato is the fourth most important crop following rice, wheat and maize (Muleta and Aga, 2019) its economic importance in developed countries and its importance as a source of nutrition in less-developed countries necessitates its continued protection from virulent phytopathogens (Campos and Ortiz, 2019). Potato, like all other crops, is subject to constant challenge from plant pathogens which can reduce both yield and revenue through both crop destruction and the infliction of deformities that are unpalatable to the consumer such as those caused by the necrotrophic fungal pathogen *Rhizoctonia solani* and up to 30% of marketable potato yield can be lost due to this pathogen (Tsror 2010). The mean annual temperatures for the South/South East of Ireland from 2017-2020 ranged from 9.8-11.1 °C for air and 9.6-11.3 °C for soil (wow.met.ie). These conditions are ideal for the development of *R. solani* pathology from both an airborne and soilborne perspective (van den Brink and Wustman, 2014) thus necessitating the use of more synthetic chemical inputs to protect plant health and maximise yield, this could apply to more just Ireland and may include other countries in Western Europe with temperate

climates. The carbon footprint of mineral fertiliser production and the ecological effect of synthetic pesticides will play ever more increasing roles in climate change and ecological/ecotoxilogical conservation targets for the global community (Brentrup et al. 2016; Panizzi et al. 2017; Siviter et al. 2018). Indeed, in Ireland an upward trend has emerged over the past number of years in the application of nitrogen, phosphorus and potassium-based (NPK) fertilisers (Fig.1) which have in turn contributed to the CO_2 equivalent emissions from agriculture (Fig. 2).

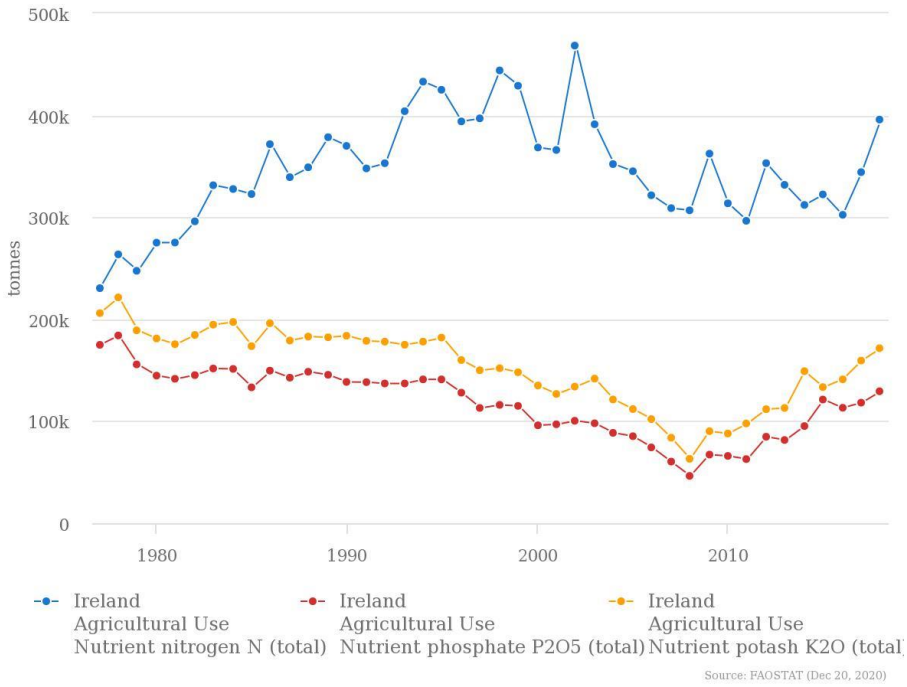


Fig.1. The agricultural use of NPK fertilisers in tonnes in Ireland from 1977-2018. Source: FAOSTAT, 2020.

Due to its large biomass in comparison to other staple crops, potato is also ideally suited to delivering additional nutrients to malnourished individuals, this concept has already been realised through the

development of the ‘Golden Potato’ with increased levels of vitamins A and E (Chitchumroonchokchai et al. 2017). With a rapidly increasing world population and changing climate, traditional methods of plant breeding will most likely fail to keep up with demand, therefore, transgenic and/or gene-editing approaches are a more enticing option, especially in regions of the world with a more open attitude to transgenic crop research.

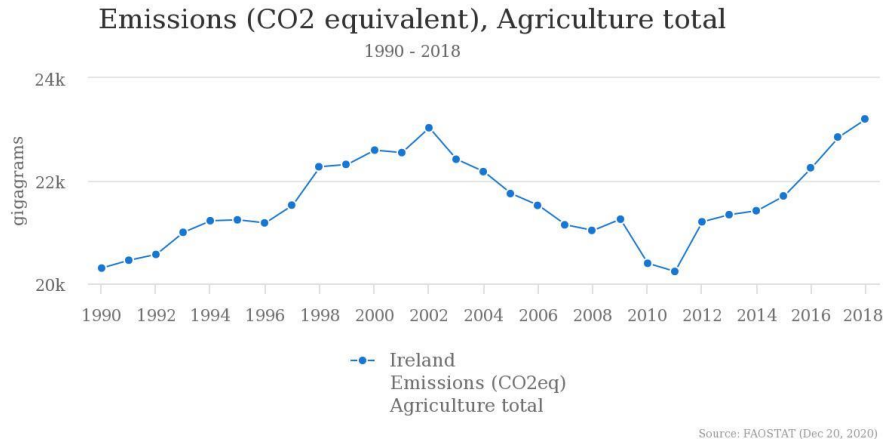


Fig. 2. Agricultural CO_2 equivalent emissions in Ireland from 1990-2018. Source: FAOSTAT, 2020.

Agrobacterium tumefaciens-mediated transformation (AMT) has been the ‘go-to’ tool for molecular plant breeding for nearly half a century and its ability to genetically transform plants has been instrumental in the development of important crops which are resistant to phytopathogens, agricultural biocides and abiotic stressors. Increasing focus has turned to investigating the role of host proteins involved in the AMT process and many excellent reviews over the past number of years have highlighted the importance that host proteins play in the

AMT process from cellular adhesion of *Agrobacterium* to its host cell and all the way down to genomic integration of the transfer DNA (T-DNA) in the host nucleus (Anand et al. 2007; Lacroix and Citovsky, 2013; Gohlke and Deeken, 2014; Gelvin, 2017; Hwang et al. 2017; Lacroix and Citovsky, 2019). The new era of genetic modification, better known as 'gene editing', has seen the emergence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) overtaking other gene editing techniques utilising zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The delivery of CRISPR/Cas gene editing complexes is often mediated by *Agrobacterium* and has been used to successfully edit crops such as tomato and wheat where induced mutations remained stable and heritable across generations (Pan et al. 2016; Zhang et al. 2019) showing that *Agrobacterium* can deliver CRISPR/Cas editing complexes to both dicots and monocots. AMT was also used to deliver the CRISPR/Cas system to field watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai). The field watermelon acetolactate synthase gene *ALS* was selected as a target for a single point mutation (C to T) which lead to increased herbicide resistance to tribenuron herbicide used to control broadleaf weeds which could compete with low-growing watermelons. Furthermore, the transgene construction was found to be absent in some T_1 plants, leading to a transgene-free herbicide resistant watermelon variety (Tian et al. 2018). Generation of transgene-free phytoene desaturase potato mutants using *Agrobacterium*-mediated CRISPR/Cas delivery has also recently been

reported (Bánfalvi et al. 2020). This may be an important step for accelerating the registration of gene edited crops in places such as the European Union in the near future.

Bacterial genotype has been shown to affect the rate of genetic transformation in diverse crops, to this end we selected two *Agrobacterium tumefaciens* genotypes namely AGL1 and EHA 105 both harbouring the same pCAMBIA transformation vector. AGL1 was selected as it has been shown previously in our lab to efficiently transform *S. tuberosum* 'Golden Wonder' (unpublished data). EHA 105 was selected as it was shown to be capable of transforming another member of the solanum family, *Solanum lycopersicum* (Tomato) at a rate of 40% and in the same study AGL1 was shown to transform tomato at a rate of 35% (Chetty et al. 2013). The objective of this study was to determine if the exposure of potato microplants to BVCs prior to undergoing AMT could possibly 'prime' the plant by modulating gene expression in the host to allow a greater rate of transient transformation efficiency. This in turn may lead to greater stable integration of genes of interest in target crops, and potentially help address our food security challenges in a more timely fashion. Additionally, BVCs of interest may be identified for future pre-treatment of plant material with the goal of increasing transformation efficiency.

Materials and Methods:

S. tuberosum Tissue Culture Maintenance:

Microplants of *S. tuberosum* L. cv. 'Golden Wonder' were supplied by the TOPS potato centre (Department of Agricultural, Food and Marine, Raphoe, Donegal, Ireland). Stocks were maintained on ½ strength Murashige and Skoog (M+S) agar consisting of: 2.2 g basal M+S medium, 15 g sucrose, 6 g agar per litre with final pH adjusted to 5.80. Nodal sections of microplants were subcultured on fresh media under aseptic conditions every four to five weeks in Microbox® 'TP 3000' growth chambers equipped with 'XXL+' filters. The microplants were kept in a growth room at 23 °C under a 16 h d^{-1} photoperiod and a photosynthetic photon flux (PPF) of 225 µmol m^{-1} s^{-1}.

Bacterial Stock Maintenance:

The commercial PGPR strain *Bacillus amyloliquefaciens* FZB24® and the Irish organic potato soil isolate *Serratia fonticola* LAC2 were maintained on tryptic soy agar (TSA). A single bacterial colony was streaked on TSA in a 90 mm Petri dish and allowed to grow overnight at 28 °C ± 2 °C or until visible colonies were observed. Cultures were sealed with Parafilm and stored at 4 °C for four to five weeks or maintained long term at -80 °C in a 50% glycerol solution.

Plant/Bacteria Co-Cultivation for BVC Exposure:

Four nodal sections of four-week-old stock plants of *S. tubersoum* L. cv. 'Golden Wonder' were placed in polyurethane foams (supplied by the School of Biological, Earth and Environmental Sciences, UCC, Ireland) in magenta containers imbibed with 50 ml autotrophic medium (½ strength M+S basal medium 2.2 g per litre, pH adjusted to 5.80). These planted magentas were placed in 'TP5000+TPD5000' Microbox® growth chambers equipped with 'XXL+' filters. These growth chambers were placed in a growth room under a 16 h photoperiod at 23 °C for two weeks to allow the microplants to acclimatise to surrounding environmental conditions. Following this the plantlets were exposed to BVCs by dropping 20 µl of each bacterial culture at OD 600nm = 1.0 on the centre of a 50 mm Petri dish containing either Methyl Red-Voges Proskeur (MR-VP) broth supplemented with 14 g agar per litre; or ½ strength M+S media containing 2.2 g M+S basal medium, 15 g sucrose and 6 g agar per litre. The bacterial inoculum was allowed to air-dry under aseptic conditions for ~15 minutes and the Petri dish was then placed inside the Microbox® growth chamber containing the microplants under aseptic conditions. The microplants were exposed to the resulting rhizobacterial BVCs, as described in chapter 4, for a period of four weeks. Control microplants were set up with either un-inoculated axenic media or no media at all. Experiment was repeated twice with four technical replicates and each technical replicate consisted of four microplants.

Generation of Transformed A. tumefaciens EHA105 and Agrobacterium-mediated transformation (AMT):

Generation of chemically competent cells and subsequent transformation of *A. tumefaciens* EHA 105 with pCAMBIA 1305.2 was carried out, with modifications, according to the Haswell Lab Manual;

(https://pages.wustl.edu/files/pages/imce/haswell/labmanual2014.final_.pdf). *Agrobacterium tumefaciens* genotypes AGL1 and EHA105 harbouring the plant transformation vector pCAMBIA 1305.2 GUSPlus^{TM} (Fig. 3) were maintained on Luria broth (LB) agar media containing the appropriate antibiotics. For AGL1 growth, LB agar media was prepared containing 50µg/ml kanamycin. For EHA105 growth, LB agar media containing 50µg/ml kanamycin and rifampicin respectively was prepared in the same manner. Following fresh sub-culturing of the respective genotypes, a single colony was selected using an inoculation loop and placed in 5 ml of liquid LB media in a 20 ml McCartney glass vial without antibiotics and allowed to grow overnight at 28°C and 170 rpm. The following morning a 3 ml starter culture of this bacterial concentrate was added to 50 ml LB media in a 250 ml conical flask. This was placed in a rotary incubator at 28°C and 220 rpm and allowed to reach an OD 600nm = 1.0. Following this 2 ml of the bacterial culture was spun down in a 2 ml centrifuge tube at 10,000 rpm for five minutes. The supernatant was subsequently poured off and the bacterial pellet was re-suspended in 1.7 ml of liquid M+S media. Roughly ~3 cm long section of stem were cut from each of the

four potato microplants, these stems were then longitudinally split using a scalpel and dipped in the respective *Agrobacterium* suspension for two minutes. Following this the stem section was blotted dry on sterile filter paper and transferred to a Petri plate containing autotrophic M+S agar and allowed to co-cultivate for four days. Histochemical staining was carried out by placing putatively transformed stems in an X-Glucuronidase (GUS) histochemical reagent mix and placed in an incubator overnight at 37 °C. Following this the stems were placed in serial 99% ethanol washes until stems were devoid of chlorophyll.

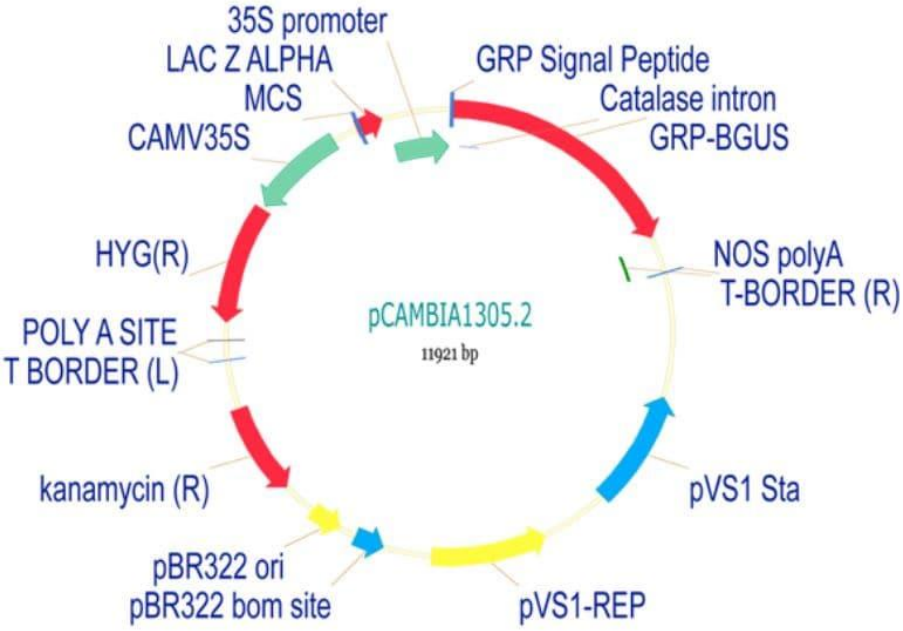


Fig. 3. Map of the pCAMBIA 1305.2 binary transformation plasmid containing the kanamycin (R) resistance to kanamycin gene for bacterial selection, the hygromycin (R) resistance to hygromycin B gene for plant selection and the GUSPlus reporter gene with catalase intron to facilitate strictly *in-planta* expression and GRP signal peptide for in-planta secretion of the GUSPlus protein.

GUS histochemical assay:

The GUS histochemical assay was prepared to a final volume of 50 ml in a sterile Corning® tube. The assay components consisted of 20mM sodium citrate buffer, 2mM potassium ferricyanide, 5 ml Triton-X-100, 5 ml methanol, 20 mg X-gluc A in a final volume of 50 ml using sterile dH_2O. The histochemical assay was filter sterilised using a 0.2 µm filter before use.

Determination of transformation rate:

Stems were imaged using an Olympus E-600 digital camera. ImageJ was used to estimate transformation rate by measuring the amount of GUS positive staining as dark blue colour against chlorotic areas of the stem. The stem section area was defined as a total count of pixels and percentage rate of transformation was calculated by measuring GUS-positive versus chlorotic areas of the stem based on their respective pixel counts.

Statistical analyses:

Transformation data was statistically analysed using SPSS 26 (IBM). Two-way analysis of variance (ANOVA) was used to determine the interaction effect of bacterial isolate and media type on plant growth. Significant differences were estimated using one-way ANOVA with Tukey test for post-hoc comparisons for normal data or Kruskal-Wallis

test for non-normal data. For normalisation of data, datasets deemed to be non-normal were transformed using square root transformations based on the distribution of scores.

Results:

To test the effect of BVCs from the two bacterial isolates, LAC2 and FZB24, on the transformation efficiency of *A. tumefaciens* AGL1 and EHA105, potato microplants were exposed to BVCs in Microbox® growth chambers followed by AMT (Fig. 4). Two-way ANOVA was used to examine the effect of BVCs emitted from isolates cultured on both media types on AGL1-mediated transformation and it revealed a non-significant effect for the interaction of isolate*media, $p = 0.036$ ($p \leq 0.01$). When MR-VP was the chosen bacterial culture media, a one-way between groups ANOVA revealed the transformation of potato stem tissue was significantly increased by BVCs emitted by LAC2 when compared to both control and FZB24 BVC treatments ($p \leq 0.001$), no significant differences were observed between FZB24 and control treatments ($p > 0.01$) (Fig. 5).

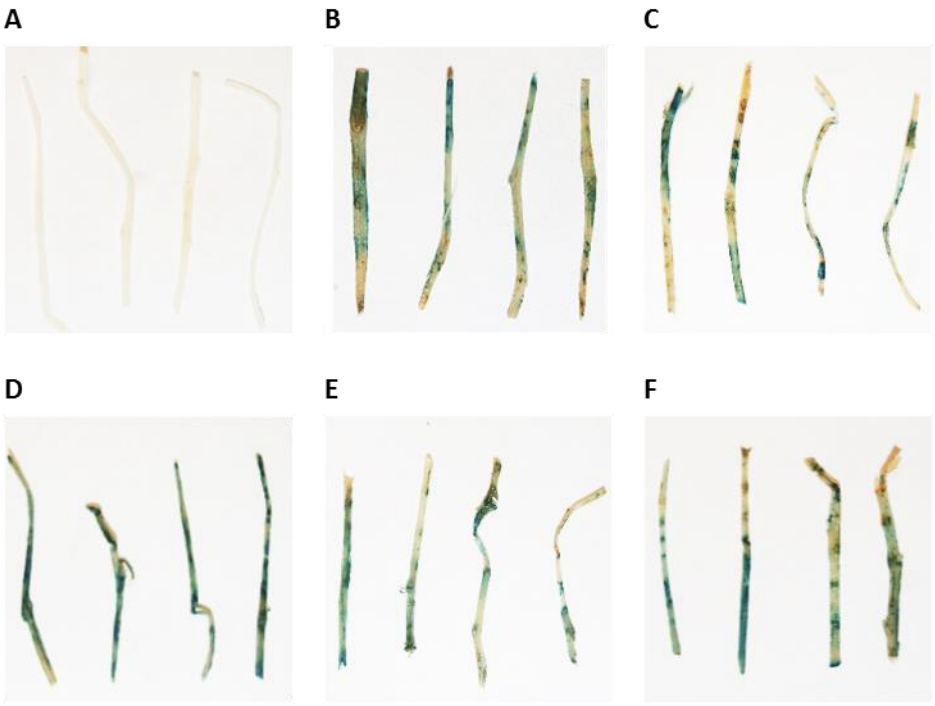


Fig. 4. *Agrobacterium tumefaciens* strain 'AGL1' transformation assay to determine transformation efficiency between VOC and non-VOC treated plants via transient GUS reporter gene expression. (A) Plants not transformed with *A. tumefaciens* or treated with BVCs. (B) Plants transformed with *A. tumefaciens* AGL1 without BVC treatment. (C) Plants treated with FZB24/MR-VP BVCs and transformed with *A. tumefaciens* AGL1. (D) Plants treated with LAC2/MR-VP BVCs and transformed with *A. tumefaciens* AGL1. (E) Plants treated with FZB24/M+S BVCs and transformed with *A. tumefaciens* AGL1. (F) Plants treated with LAC2/M+S BVCs and transformed with *A. tumefaciens* AGL1.

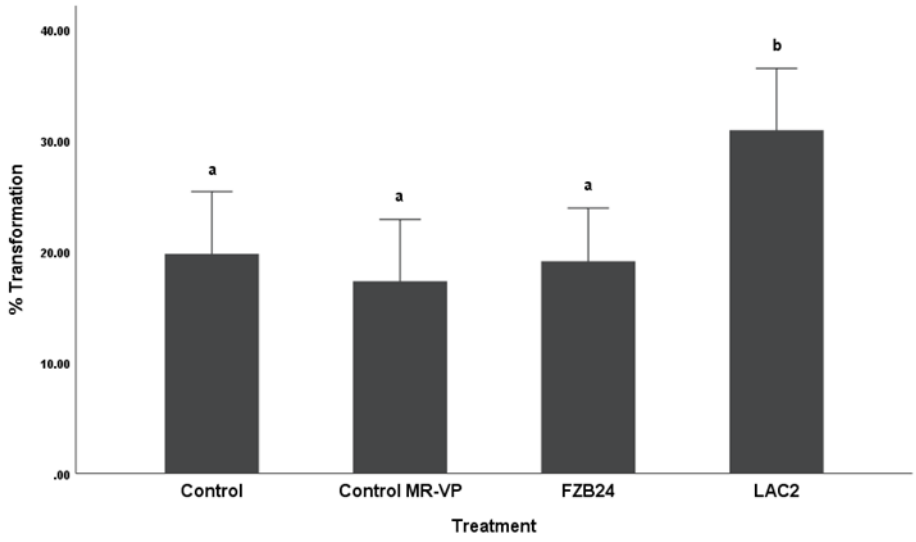


Fig. 5. Demonstration of MR-VP-derived BVC-mediated effect on genetic transformation of potato by *A. tumefaciens* genotype AGL1. Treatments which share a common letter are not significantly different to one another ($p > 0.01$) according to one-way between groups ANOVA with Tukey post hoc test. Error bars represent confidence interval of the mean (99%).

When M+S media was the chosen bacterial growth medium, the AGL1-mediated transformation rate was diminished in comparison to BVCs emitted from MR-VP derived cultures. Again, a one-way between groups ANOVA revealed that LAC2 BVCs displayed significant differences, but only when compared to the control M+S treatment ($p < 0.01$). There were no significant differences in transformation rate between FZB24 and both controls or between LAC2, control and FZB24 treatments ($p > 0.01$) (Fig. 6).

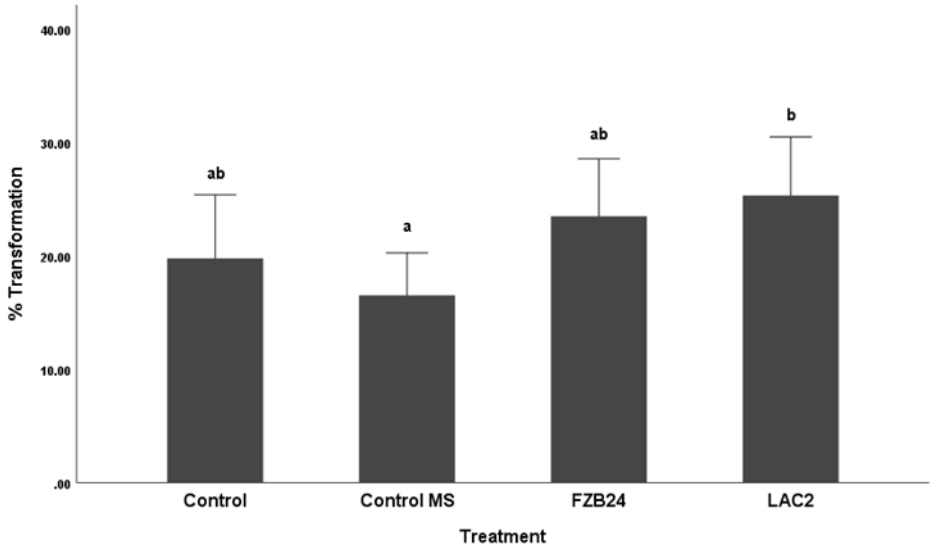


Fig. 6. Demonstration of M+S-derived BVC-mediated effect on genetic transformation of potato by *A. tumefaciens* genotype AGL1. Treatments which share a common letter are not significantly different to one another ($p > 0.01$) according to one-way between groups ANOVA with Tukey post hoc test. Error bars represent confidence interval of the mean (99%).

Marked differences were observed in EHA105 transformation rates between treatment groups when isolates were cultured on MR-VP agar. Both of the BVC treatments were significantly different from both controls ($p < 0.001$), however neither the members of the control treatments nor the members of the BVC treatments were different from

one another ($p > 0.01$). There was a 6- and 8-fold increase in transformation rate between the control treatment and FZB24 and LAC2 treatments respectively (Fig. 7). Interestingly, a similar trend in EHA105 transformation rates was observed when FZB24 and LAC2 were cultured on M+S agar (Fig. 8). The transformation rate after BVC pre-treatment from LAC2 was an average of 41% when cultured on both media types, whereas FZB24 was 33% and 30% when cultured on MR-VP and M+S respectively, indicating that at least in the case of EHA105, a shared volatile(s) emitted from both isolates on both media types may be responsible for the observed similarities in transformation rate trends between the two treatments. The control with axenic M+S media transformation frequency (23.5%) was over twice that of the control with axenic MR-VP treatment (9%) this may be accounted for by other abiotic factors encountered by the plant during growth.

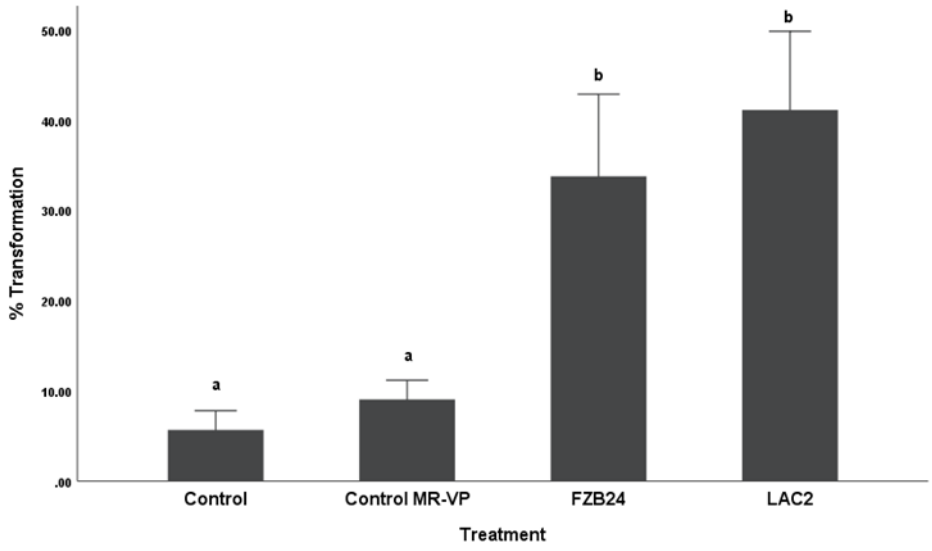


Fig. 7. Demonstration of MR-VP-derived BVC-mediated effect on genetic transformation of potato by *A. tumefaciens* genotype EHA105. Treatments which share a common letter are not significantly different to one another ($p > 0.01$) according to independent samples Kruskal-Wallis test with Bonferroni correction for multiple tests. Error bars represent confidence interval of the mean (99%).

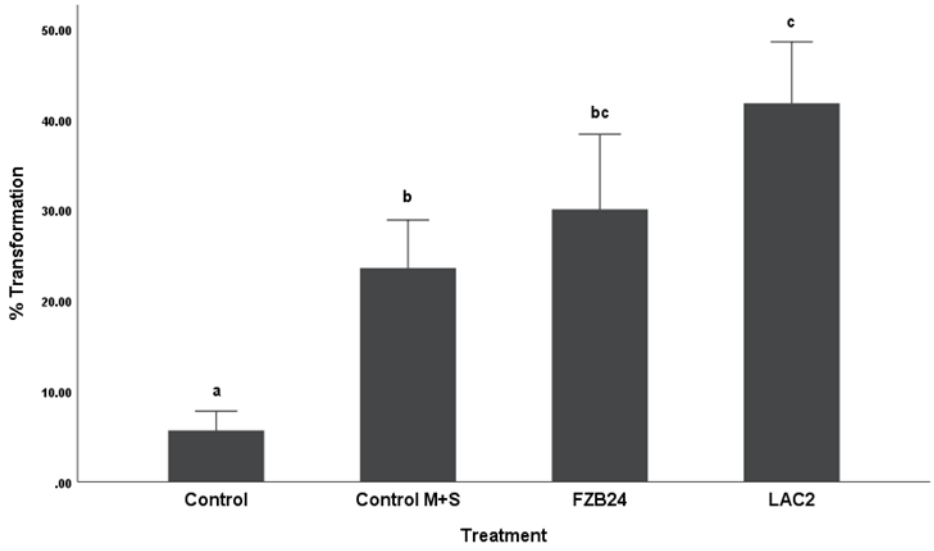


Fig. 8. Demonstration of M+S-derived BVC-mediated effect on genetic transformation of potato by *A. tumefaciens* genotype EHA105. Treatments which share a common letter are not significantly different to one another ($p > 0.01$) according to independent samples Kruskal-Wallis test with Bonferroni correction for multiple tests. Error bars represent confidence interval of the mean (99%).

Discussion:

The use of microbes for sustainable agriculture has been the focus of intense research for the past number of decades as humanity begins to come to terms with the effects of our intensive activities on the finely balanced ecosystems of Earth. The exploitation of bacterial PGP activities has been particularly prevalent, one of these activities is the production of volatile organic compounds which have been shown to have both direct and indirect effects on plant growth. Indirect effects include those associated with biocidal effects towards phytopathogenic organisms thus prohibiting them from developing a disease state in the target host whereas direct volatile-mediated effects on plant growth concern the alteration of gene expression in the volatile-sensor plant in response to a single, or blend of, volatile organic compounds. The

alterations in gene expression often concern those associated with defence or stress responses.

Agrobacterium tumefaciens, the causative agent of crown gall disease in many dicotyledonous plants has been widely used in biotechnological applications over the past five decades to introduce genes of interest into many crop plants in order to improve their defence against agricultural pests, diseases and abiotic stressors. This technology can have profound effects for the way we feed our planets population and can potentially decrease our need to employ more environmentally-destructive conventional agricultural practices. One of the main stumbling blocks in the use of AMT has been the often low amount of transient and, in particular, stably transformed plants which can be used for further study and/or transfer to the field post-AMT. This has been historically prevalent in the monocotyledonous plants such as wheat and barley (Singh and Prasad, 2016) however there has been a significant success story in the transformation of monocots through the development of *Zea mays* event MON810, which has been grown in many EU countries since its initial authorization for commercial cultivation in 1998 (Bertho et al. 2020).

Numerous studies have attempted to use both biochemical and abiotic inputs to AMT systems in order to increase transformation efficiencies. The plant signalling molecule, acetosyringone, can be applied exogenously to transformation systems in order to increase AMT efficiency (Godwin et al. 1991), additionally alterations in pH, temperature, co-cultivation parameters and photoperiod can have

profound effects on transformation efficiency (Rai et al. 2012; Zambre et al. 2003). Recently, chemical alterations during *A. tumefaciens* EHA105 culture through the addition of 100 μM dithiothreitol, 20 μM azacitidine, 5 mM spermidine, and 120 μM acetosyringone were shown to induce an 8-fold increase in GUS activity compared to the control, demonstrating that not only pre-treatment of plant material but also pre-treatment of *Agrobacterium* can increase its T-DNA transfer rates (Zhao et al. 2020). Potato explants exposed to BVCs in an I-plate system displayed increased transformation rates in potato with *Agrobacterium* and *Rhizobium leguminosarum* bv. Trifoli ANU845-mediated transformation resulting in a 5-and 7-fold increase in transformation respectively (Doyle Prestwich and O' Herlihy, 2011). We developed a novel co-cultivation system for the sustained exposure of whole potato microplants to BVCs from both a commercially available bacterial isolate (FZB24) and an Irish potato soil isolate (LAC2) cultured on two different media types namely, MR-VP and M+S, for a period of four weeks.

Following plant exposure to BVCs the subsequent plant transformation rates using strains AGL1 and EHA105 displayed marked differences in transformation efficiencies depending on BVC-emitting bacteria, their growth media and/or the genotype of *A. tumefaciens*. For AGL1-transformed plants, LAC2 consistently had higher transformation efficiency than both FZB24 and control treatments, especially when cultured on MR-VP, where significant differences were observed between LAC2 and the other treatments. When isolates were cultured

on M+S this effect diminished and no significant difference was observed in transformation efficiency between LAC2, FZB24 and the control treatment. Again, for EHA105-transformed plants on the whole, there were significant differences observed between BVC-treated plants and controls. Interestingly, transformation efficiency was often higher among EHA105-transformed plants than that of AGL1-transfomed plants, suggesting a possible genotype-dependent effect. Nonetheless, BVC-treated plants broadly maintained higher transformation efficiencies. Interestingly, similar transformation rates were observed for these *Agrobacterium* strains when transforming *Solanum lycopersicum* L. cv. Micro-Tom, however not in a system investigating BVC-mediated effects (Chetty et al. 2013). As described in chapter 4, when cultured in MR-VP, LAC2 emits a blend of 15 unique BVCs in comparison to FZB24, in FZB24 a blend of five unique BVCs are emitted in comparison to LAC2 and both isolates share a blend of three common BVCs (Table 1).

Table 1. Unique and shared BVCs between LAC2 and FZB24 when cultured on MR-VP media

LAC2 unique BVCs	FZB24 unique BVCs	Shared BVCs
3-methyl-1-butanol	3-hydroxy-2-butanone	Hexamethylcyclotrisiloxane
Dimethylamine	2,3-butnaediol	2-piperidinone
2-butanamine	Dodecane	Hexadecane
1-decanol	2-ethyl-1-hexanol	
1-dodecanol	Hydroxyurea	
2-ethyl-1-pentanol		
1-heptadecanol		
2-nonaone		
Cyclopropyl carbinol		
Pentanol		
Octanal		
Hexadecanal		
5-methylundecane		
2-undecanone		
2-heptadecanone		
2-tridecanone		
15	**5**	**3**

Modified from table 1, chapter 4.

When cultured in M+S just 7 unique BVCs are observed in LAC2, none are observed in FZB24 and just one, hexamethylcyclotrisiloxane, are shared between both isolates (Table 2). This volatile may be an interesting target for exogenous pre-treatment of plants selected for AMT in future as it's commonality between both isolates may indicate a favourable activity towards transformation efficiency. Interestingly,

when cultured on nutrient agar medium, all 49 endophytic bacterial isolates of sugarcane in a recent study on the biocontrol of *Colletotrichum falcatum* also produced hexamethylcyclotrisiloxane (Jayakumar et al. 2020), this BVC was also identified in the volatile blend of the biocontrol strain *Bacillus subtilis* Bs-1 (Cao et al. 2019). While hexamethylcyclotrisiloxane may be an interesting target, it alone cannot explain the consistently superior effect of the LAC2 volatile blend on transformation efficiency as FZB24 also emits hexamethylcyclotrisiloxane. The superior effect may instead be induced by other unique volatiles such as 2-nonanone, 2-undecanone, 2-heptadecanone and 2-tridecanone which are prime targets for future analyses of AMT using pure synthetic volatiles as plant pre-treatments either singly or as a variety of BVC blends.

Table 2. Unique and shared BVCs between LAC2 and FZB24 when cultured on M+S media

LAC2 unique BVCs	**FZB24 unique BVCs**	**Shared BVCs**
3-methyl-1-butanol	N/A	Hexamethylcyclotrisiloxane
2-undecanone		
2-heptadecanone		
2-tridecanone		
2-nonanone		
2-methyl-1-butanol		
3-methylbutanal		
7	**0**	**1**

Modified from table 1, chapter 4.

Our analysis of differentially expressed genes (DEGs) post-exposure to BVCs in chapter four revealed a number of defence and/or stress-related DEGs which were downregulated. Both isolate BVCs were capable of the common downregulation of defence genes such as *EDS1* (discussed in chapter four) however there were a greater number of unique defence genes downregulated via LAC2 BVC exposure. Two genes, *MPK3* and *PEN1*, may play crucial roles in mediating the increased transformation efficiency of LAC2 BVCs. MPK3 is involved in normal plant growth and development and also in the downstream events involved in defence gene activation after recognition of a pathogen associated molecular pattern (PAMP) (Colcombet and Hirt, 2008). While MPK3 downregulation prior to AMT may initially help *Agrobacterium* to transform the cell, any subsequent upregulation of MPK3 after this event may actually increase transformation rates via hijacking of the host VIP1 protein involved in T-DNA nuclear integration, as VIP1 is also an activator of defence genes it is targeted by the *Agrobacterium* VirF protein for proteasomal degradation (Pitzschke et al. 2009; Citovsky et al. 2004; Djamei et al. 2007; Tzifra et al. 2004).

A number of other genes are involved in defence responses such as BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1), beta-glucan-binding protein 4 and WRKY transcription factors, all of which are downregulated in LAC2 BVC-treated plants.

Brassinosteroids are a group of steroidal compounds specific to plants which are essential for normal growth and development. Additionally

they are also involved in responses to to different stressors such as interactions with pathogens. BAK1 along with its co-receptor BR-INSENSITIVE 1 (BRI1) are leucine rich repeat-receptor like kinases (LRR-RLKs) located in the plasma membrane. Upon brassinosteroid binding the BRI1 kinase is activated by autophosphorylation and BAK1 transphosphorylation, during pathogen recognition this activates signalling cascades associated with bacterial defence responses via flg22 (flagellin 22) binding, or alternatively, microbe-associated molecular pattern or pathogen-associated molecular pattern (MAMP/PAMP)-triggered immunity via binding of elf18 (elongation factor 18) to BAK1 (Yu et al. 2018). In the LAC2 BVC-treated plants, BAK1 is downregulated compared to control plants, suggesting that one or more LAC2 BVCs may be used for immunomodulation of plant hosts.

During plant colonisation the cell wall is the initial microbial structure that either makes direct physical contact with the host cell or alternatively through the release of oligomers mediated by a variety of plant-secreted hydrolases (Rovenich et al. 2016). β-glucans make up the majority of the fungal cell wall architecture (Brown and Gordon, 2003). In recent years the role of β-glucans acting as potential MAMPs has been investigated, one such study investigates the interactions between plants and *Piriformospora indica* (Basidomycota, Sebacinales). The root fungal endophyte *P. indica* is a symbiont which colonises, intra- and intercellulary, the root and cortex cells of the roots of a wide variety of unrelated plants, including *A. thaliana* and

Horduem vulgare dicotyledonous and monocotyledonous plants respectively. *P. indica* employs a biphasic lifecycle, the first phase is biotrophic followed by a stage where the symbiont is often found in dead or dying cells of the host where it secretes hydrolytic enzymes that break down plant cell walls and proteins (Lahrmann et al. 2013). A small fungal specific protein called, Fungal Glucan-Binding 1 (FGB1), was found to be induced during colonisation of *A. thaliana* and *H. vulgare* by *P. indica.* By biochemical and cytological analyses it was shown that FGB1 is a secreted lectin which binds β-glucan, alters cell wall composition of the fungus and suppresses glucan-triggered reactive oxyegen species production in different plants thus effecting fungal colonisation (Wawra et al. 2016). Again this may be a microbial pre-colonisation effect mediated by one or more BVCs emitted by LAC2.

In potato, WRKY family transcription factors (TF) are an important group of transcription factors, but are also widely employed among many plant families (Chacón-Cerdas et al. 2020). WRKY transcription factors act as activators, repressors or co-repressors of many important pathways such as those involved in the production of alkaloids (Schluttenhofer and Yuan, 2015) and the activation of defence pathways (Ishihama and Yoshioka, 2012). A WRKY transcription factor (NtWRKY8), downregulated by LAC2 BVC exposure described in chapter 4, has been functionally assessed in potato by Yogendra et al. and they found this transcription factor is expressed in the presence of *P. infestans* and is responsible for the regulation of

benzylisoquinoline alkaloid pathway genes. StWRKY8 binds to the W-box domain on the promoter of resistance-related metabolite genes that encode for the biosynthetic enzymes: codeinone reductase 2-like, tyrosine decarboxylase and noroclaurine synthase. These induce the accumulation of metabolites involved in cell wall reinforcement such as morphine-3-glucuronide and morphine-6-glucuronide which are involved in cross-linking pectins and resiatnce to pectinase (Yogendra et al. 2017).

SWEET (Sugars Will Eventually be Transported) genes encode transporter proteins and often function as susceptibility (S) genes. The recessive alleles of SWEET genes confer resistance to pathogens. S genes follow an inverse gene-for-gene model, where the virulence gene of the pathogen can only cause infection when the host is carrying a dominant allele of an S gene. During infection, pathogens introduce TALE (Transcription activator-like) effectors into the host cytoplasm, TALEs then make their way to the cytoplasm where they activate the expression of SWEET genes and the resulting sugars make their way for export to the apoplast where they feed the pathogen (Gupta et al. 2021). UPA16, which has been shown in chapter 4 to be upregulated in response to LAC2 BVCs, is a SWEET gene. It was first identified by Kay et al. in pepper where it functions as an S gene upon infection by *Xanthomonas campestris pv. vesicatoria* the causal pathogen for the bacterial disease, leaf streak. However, the precise role of UPA16 as an S gene in leaf streak of pepper has not been fully elucidated (Kay et al. 2009). It could be that LAC2 BVCs act to upregulate sugar transport

in potato to act as a carbon source for the nutritive benefit of this beneficial bacterium.

PEN1 is a "frontline" defender involved in resisting the penetration of phytopathogens into the plant cell (Collins et al. 2003) and downregulation in this gene may lead to more efficient transfer of T-DNA to the cytoplasmic space via the T4SS. Upregulation of a defence-related gene *PSK*, which encodes for a growth-promoting peptide hormone was observed in LAC2 BVC-treated plants. PSK is involved in the attenuation of pattern-triggered immunity following plant recognition of microbe associated molecular-patterns (MAMPs) in order to negate the effects of chronic and/or intense immune activation in plants which can ultimately be detrimental to plant health (Igarishi et al. 2012). Application of PSK to carrot hypocotyls resulted in a five-fold increase in AMT after four weeks of culture, this was attributed to enhanced cell proliferation via PSK signalling leading to an increased transformation efficiency (Matsubayashi et al. 2004). Further manipulation of the plant immune response using the phytopathogenic effector AvrPto from the plant pathogen *Pseudomonas syringae* also resulted in increased AMT efficiency. When conditionally expressed in *Arabidopsis* under the control of a dexamethasone (DEX) inducible promoter, AvrPto greatly increased the transient transformation rate of *Agrobacterium* when plants were pre-treated with a DEX spray prior to infection. Confirmation of the effect of AvrPto was established when DEX pre-treatment resulted in no increase of GUS expression in Col-0 or other non-AvrPto transgenic

plants analysed in this study (Tsuda et al. 2012). These observations demonstrate that manipulations of the plant immune response are ideal pre-treatment targets which can allow *Agrobacterium* to more effectively colonise the host and thus increase both transient and as a result, stable transformation rates.

Taken together, the up-and downregulation of defence and/or stress-related genes in FZB24 and particularly LAC2 BVC-treated plants can contribute substantially to the interpretation of increased transformation efficiencies via AMT through our growth system (Fig. 9).

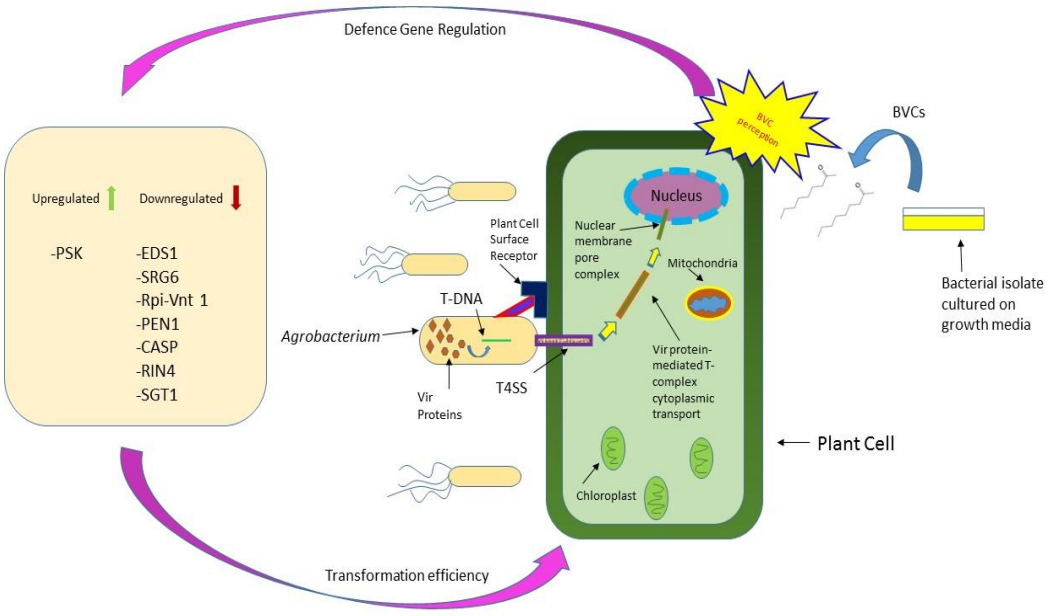


Fig. 9. Schematic demonstrating a simplified series of the events involved in AMT and the effect that BVCs may have on transformation efficiency via differential expression of a number of *S. tuberosum* defence genes post-exposure to BVCs in Microbox growth chambers. BVC perception by the plant cell is still not fully understood with regard to receptors and associated pathways of BVC signals.

Further work such as mutational analysis and application of pure volatiles will be needed to copper-fasten these observations in potato. It may be the case that *Agrobacterium* can take advantage of these volatile-mediated effects in the wild, where crosstalk between microorganisms in the soil may induce volatile-mediated differential gene expression in host plants which *Agrobacterium* may exploit to effectively transfer its T-DNA and/or enter the host organism itself. The interplay between plant defence and *Agrobacterium* is discussed in-depth in two excellent reviews (Hwang et al. 2015; Pitzschke 2013). Interestingly the BVCs 2-nonanone, 2-heptanone, 2-undecanone were recently shown to inhibit the growth of *Agrobacterium* biofilms from strains Sh-1 and Chry 5 wild type strains in I-plate assays after 75 h (Plyuta et al. 2016), these volatiles were also identified in the headspace of LAC2 in our study and crucially, in our experimental setup, plants are pre-treated with the BVCs 2-nonanone, 2-heptanone and 2-undecanone, among others, however only the plants are exposed to these BVCs, not the *Agrobacterium* therefore it could be hypothesised that plant recognition of these volatiles may initiate an evolutionarily conserved mechanism whereby defence genes are downregulated in the presence of beneficial VOC's that inhibit the growth of pathogenic microorganisms, an observation worthy of further investigation. As our understanding of the highly complex matrix of interactions between volatiles, blends of volatiles and their implications in crosstalk amongst a great deal of organisms within different biomes we may be able to exploit these molecules for both

sustainable agriculture in the classical sense and biotechnological applications to better feed our planets population and greatly minimise our impact on the global ecosystem as a whole.

References:

Anand A, Vaghchhipawala Z, Ryu CM, Kang L, Wang K, del-Pozo O, Martin GB, Mysore KS (2007) Identification and characterization of plant genes involved in *Agrobacterium*-mediated plant transformation by virus-induced gene silencing. Mol Plant Microbe Interac 20:41-52

Bánfalvi Z, Csákvári E, Villányi V, Kondrák M (2020) Generation of transgene-free PDS mutants in potato by *Agrobacterium*-mediated transformation. BMC Biotechnol 20:1-10

Bertho L, Schmidt K, Schmidtke J, Brants I, Cantón RF, Novillo C, Head G (2020) Results from ten years of post-market environmental monitoring of genetically modified MON 810 maize in the European Union. PLoS One 15:e0217272

Brown GD, Gordon S (2003) Fungal β-glucans and mammalian immunity. Immunity 19:311-315.

Campos H, Ortiz O (2020) The Potato Crop: Its Agricultural, Nutritional and Social Contribution to Humankind. Springer Nature

Cao H, Jiao Y, Yin N, Li Y, Ling J, Mao Z, Yang Y, Xie B (2019) Analysis of the activity and biological control efficacy of the *Bacillus subtilis* strain Bs-1 against *Meloidogyne incognita*. Crop Prot 122:125-135

Chacón-Cerdas R, Barboza-Barquero L, Albertazzi FJ, Rivera-Mendez W (2020) Transcription factors controlling biotic stress response in potato plants. Physiol Mol Plant Path 8:101527.

Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML (2013) Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. Plant Cell Rep 32:239-247

Chitchumroonchokchai C, Diretto G, Parisi B, Giuliano G, Failla ML (2017) Potential of golden potatoes to improve vitamin A and vitamin E status in developing countries. PLoS One 12:e0187102

Colcombet J, Hirt H (2008) *Arabidopsis* MAPKs: a complex signalling network involved in multiple biological processes. Biochem J 413:217-226

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425:973-977

Citovsky V, Kapelnikov A, Oliel S, Zakai N, Rojas MR, Gilbertson RL, Tzfira T, Loyter A (2004) Protein interactions involved in nuclear import of the *Agrobacterium* VirE2 protein in vivo and in vitro. J Biolo Chem 279:29528-29533

Djamei A, Pitzschke A, Nakagami H, Rajh I, Hirt H (2007) Trojan horse strategy in *Agrobacterium* transformation: abusing MAPK defense signaling. Science 318:453-456

Doyle Prestwich B, O'Herlihy E (2011) A preliminary investigation into how plant growth promoting rhizobacteria can influence the frequency of gene transfer in *Agrobacterium*- and Transbacter™ mediated transformation systems. In: VII International Symposium on In Vitro Culture and Horticultural Breeding 961:109-114

Garnett T (2014) Three perspectives on sustainable food security: efficiency, demand restraint, food system transformation. What role for life cycle assessment? J Clean Prod 73:10-18

Gelvin SB (2017) Integration of Agrobacterium T-DNA into the plant genome. Ann Rev Gen 51:195-217

Godwin I, Todd G, Ford-Lloyd B, Newbury HJ (1991) The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. Plant Cell Rep 9:671-675

Gohlke J, Deeken R (2014) Plant responses to *Agrobacterium tumefaciens* and crown gall development. Front Plant Science 5:155

Gupta PK, Balyan HS, Gautam T (2021) SWEET genes and TAL effectors for disease resistance in plants: Present status and future prospects. Mol Plant Pathology 22:1014-1026

Heenan-Daly D, Velivelli SL, Prestwich BD (2019) The Role of Rhizobacterial Volatile Organic Compounds in a Second Green Revolution—The Story so Far. In Field Crops: Sustainable Management by PGPR 2019, pp. 191-220. Springer, Cham

Hwang EE, Wang MB, Bravo JE, Banta LM (2015) Unmasking host and microbial strategies in the *Agrobacterium*-plant defense tango. Front Plant Sci 6:200

Hwang HH, Yu M, Lai EM (2017) *Agrobacterium*-mediated plant transformation: biology and applications. The Arabidopsis Book 15

Ishihama N, Yoshioka H (2012) Post-translational regulation of WRKY transcription factors in plant immunity. Curr Opin Plant Biol 15:431-437.

Jayakumar V, Sundar AR, Viswanathan R (2020) Biocontrol of *Colletotrichum falcatum* with volatile metabolites produced by endophytic bacteria and profiling VOCs by headspace SPME coupled with GC–MS. Sugar Tech 16:1-4

Jiang CH, Xie YS, Zhu K, Wang N, Li ZJ, Yu GJ, Guo JH (2019) Volatile organic compounds emitted by *Bacillus* sp. JC03 promote plant growth through the action of auxin and strigolactone. Plant Growth Regul 87:317-328

Kay S, Hahn S, Marois E, Wieduwild R, Bonas U (2009) Detailed analysis of the DNA recognition motifs of the *Xanthomonas* type III effectors AvrBs3 and AvrBs3Δrep16. Plant J 59:859-871.

Lacroix B, Citovsky V (2013) The roles of bacterial and host plant factors in *Agrobacterium*-mediated genetic transformation. Int J Dev Biol 57:467-481

Lacroix B, Citovsky V (2019) Pathways of DNA transfer to plants from *Agrobacterium tumefaciens* and related bacterial species. Ann Rev Phytopath 57:231-251

Lahrmann U, Ding Y, Banhara A, Rath M, Hajirezaei MR, Döhlemann S, von Wirén N, Parniske M, Zuccaro A (2013) Host-related metabolic cues affect colonization strategies of a root endophyte. Proc Natl Acad Sci U.S.A 110:13965-13970.

Matsubayashi Y, Goto T, Sakagami Y (2004) Chemical nursing: phytosulfokine improves genetic transformation efficiency by promoting the proliferation of surviving cells on selective media. Plant Cell Rep 23:155-158

Morath SU, Hung R, Bennett JW (2012) Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. Fungal Biol Rev 26:73-83

Moretti A, Pascale M, Logrieco AF (2019) Mycotoxin risks under a climate change scenario in Europe. Trends Food Sci Technol 84:38-40.

del Carmen Orozco-Mosqueda M, Macías-Rodríguez LI, Santoyo G, Farías-Rodríguez R, Valencia-Cantero E (2013) *Medicago truncatula* increases its iron-uptake mechanisms in response to volatile organic compounds produced by *Sinorhizobium meliloti*. Folia Microbiol 58:579-585

Muleta HD, Aga MC (2019) Role of nitrogen on potato production: a review. J Plant Sci 7:36-42

Pan C, Ye L, Qin L, Liu X, He Y, Wang J, Chen L, Lu G (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. Sci Rep 6:24765

Panizzi S, Suciu NA, Trevisan M (2017) Combined ecotoxicological risk assessment in the frame of European authorization of pesticides. Sci Total Environ 580:136-146

Pitzschke A (2013) *Agrobacterium* infection and plant defense—transformation success hangs by a thread. Front Plant Sci 4:519

Plyuta V, Lipasova V, Popova A, Koksharova O, Kuznetsov A, Szegedi E, Chernin L, Khmel I (2016) Influence of volatile organic compounds emitted by *Pseudomonas* and *Serratia* strains on *Agrobacterium tumefaciens* biofilms. Apmis 124:586-594

Rai GK, Rai NP, Kumar S, Yadav A, Rathaur S, Singh M (2012) Effects of explant age, germination medium, pre-culture parameters,

inoculation medium, pH, washing medium, and selection regime on *Agrobacterium*-mediated transformation of tomato. In Vitro Cell Dev Biol-Plant 48:565-578

Rovenich H, Zuccaro A, Thomma BP (2016) Convergent evolution of filamentous microbes towards evasion of glycan-triggered immunity. New Phytol 212:896-901.

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017-1026

Schluttenhofer C, Yuan L (2015) Regulation of specialized metabolism by WRKY transcription factors. Plant Physiol 167:295-306.

Singh RK, Prasad M (2016) Advances in *Agrobacterium tumefaciens*-mediated genetic transformation of graminaceous crops. Protoplasma 253:691-707

Siviter H, Koricheva J, Brown MJ, Leadbeater E (2018) Quantifying the impact of pesticides on learning and memory in bees. J Appl Ecol 55:2812-2821

Tian S, Jiang L, Cui X, Zhang J, Guo S, Li M, Zhang H, Ren Y, Gong G, Zong M, Liu F (2018) Engineering herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing. Plant Cell Rep 37:1353-1356

Tsror L (2010) Biology, epidemiology and management of *Rhizoctonia solani* on potato. J Phytopath158:649-658

Tsuda K, Qi Y, Nguyen LV, Bethke G, Tsuda Y, Glazebrook J, Katagiri F (2012) An efficient *Agrobacterium*-mediated transient transformation of *Arabidopsis*. Plant J 69:713-719

Tzfira T, Vaidya M, Citovsky V (2004) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature 431:87-92

Vlassi A, Nesler A, Perazzolli M, Lazazzara V, Büschl C, Parich A, Puopolo G, Schuhmacher R (2020) Volatile organic compounds from *Lysobacter capsici* AZ78 as potential candidates for biological control of soilborne plant pathogens. Front Microbiol 11:1748

Wang J, Zhou C, Xiao X, Xie Y, Zhu L, Ma Z (2017) Enhanced iron and selenium uptake in plants by volatile emissions of *Bacillus amyloliquefaciens* (BF06). Appl Sci 7:85

Wawra S, Fesel P, Widmer H, Timm M, Seibel J, Leson L, Kesseler L, Nostadt R, Hilbert M, Langen G, Zuccaro A (2016) The fungal-specific β-glucan-binding lectin FGB1 alters cell-wall composition and suppresses glucan-triggered immunity in plants. Nat Commun 7:1-11.

Yogendra KN, Dhokane D, Kushalappa AC, Sarmiento F, Rodriguez E, Mosquera T (2017) StWRKY8 transcription factor regulates

benzylisoquinoline alkaloid pathway in potato conferring resistance to late blight. Plant Sci 256:208-216.

Yu MH, Zhao ZZ, He JX (2018) Brassinosteroid signaling in plant–microbe interactions. Int J Mol Sciences 19:4091.

Zambre M, Terryn N, De Clercq J, De Buck S, Dillen W, Van Montagu M, Van Der Straeten D, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta 216:580-586

Zhao H, Jia Y, Cao Y, Wang Y (2020) Improving T-DNA Transfer to *Tamarix hispida* by Adding Chemical Compounds During *Agrobacterium tumefaciens* Culture. Front Plant Sci 11:1490

General Conclusion and Future Perspectives

More sustainable solutions for agriculture are needed if a secure global food supply is to be maintained in the face of climate change and ecological destruction brought about in a large part due to agriculture (Fellmann et al. 2018). The Food and Agriculture organisation of the United Nations (FAO) has recently released their report on the status of food security and nutrition in the world for 2020 and state a number of findings. The reports main findings highlight that ~2 billion people worldwide did not have regular access to safe, nutritious and sufficient food in 2019. The target of the Sustainable Development Goal (SDG) #2 'Zero Hunger' by 2030, if recent trends continue, will not be met and the number of people affected by hunger will likely stretch beyond 840 million by 2030 and furthermore, the COVID-19 pandemic may add anywhere from 83-132 million people to the total number of undernourished in the world in 2020. In 2019, 21.3% (144 million) of children under 5 years of age were stunted and 6.9% (47 million) were wasted. Unsurprisingly, the emergence of the COVID-19 pandemic has presented challenges for vulnerable communities as food supply chains can be disrupted, restrictions to peoples movements can affect access to markets, price increases for food (nutritious food in particular) coupled with possible job losses can minimise access to adequate nutrition and can consequently increase the risk of malnutrition (FAO, 2020; IPCC 2021). As a result of this, people's adjusted spending patterns favour products with a longer shelf life and often poorer nutrition profiles (IFIC, 2020). Global crises can affect the delivery of

nutritious food to the most vunerable people on the planet, those who are at serious risk of malnutrition. One of the hidden costs of this, besides that to human health, is in relation to the climate-related consequences of our diets and the systems that support these. The diet-related social cost of greenhouse gas emissions associated with today's dietary patterns is estimated to be more than USD 1.7 trillion per year by 2030 and the FAO states that countries will need a rebalancing of agricultural policies and incentives towards more nutrition-sensitive investment and policy actions all along the food supply chain to reduce food losses and enhance efficiencies at all stages (FAO, 2020).

The work contained in this thesis has identified rhizobacteria with plant growth-promoting potential by carrying out an isolation campaign in Irish potato soils, both organic and conventional, for bacterial biochemical traits that can contribute to sustainable agriculture. These are, the production of ammonia, hydrogen cyanide and siderophores, the ability to solubilise tricalcium phosphate and in particular, the emission of blends of volatile organic compounds with agronomic interest that can both promote plant growth of potato in vitro and inhibit the growth of a phytopathogen, in this case *Rhizoctonia solani*. The exploration of these volatiles for biotechnological applications is also assessed through the enhancement of *Agrobacterium tumefaciens*-mediated transformation of potato which has been exposed to bacterial volatiles prior to undergoing transformation, displaying the breadth of applications that these molecules can address. Extensive exploration of the literature has highlighted the foundations which support these areas

of plant-microbe research and discusses their early findings and current trends, historic contributions to sustainable agriculture through in vitro lab studies and in vivo field applications, and the future potential which these microbes possess to enhance sustainable agriculture across virtually all sectors of the industry.

Notwithstanding people's attitudes and habits towards food consumption in the developed world, we are now in dire need of sustainable solutions for food production, and particularly in developing countries, the need to increase its nutritional content. **Chapter 1** examines how this can be addressed through the use of PGP microbes in the biofortification of crop plants, this can help alleviate 'hidden hunger', a condition where someone can have an adequate food supply, at least enough to feel satiated, but can be lacking in micronutrients important for optimal health such as Se, Fe and Zn. The use of PGP microbes for biofortification can address a number of the United Nations' 17 SDGs, for example SDG #2 'Zero Hunger'; #3 'Good Health and Well-Being'; #12 'Responsible Consumption and Production'; #13 'Climate Action' and #15 'Life on Land'. The uptake of micronutrients such as Fe and Zn can be greatly increased by co-application of PGP microbes and micronutrient amendments to agricultural inputs as evidenced by Rana et al. (2015), when studied in rice, a bacterial-cyanobacterial consortia increased Fe concentration from ~4 to 14% over the fertilizer-only control and furthermore a single isolate *Providencia* sp. PW5 increased the Fe concentration by ~45% compared to the fertilizer-only control in wheat grains (Rana et al.

2015). Manjunath et al. (2016) recorded increased Zn concentration in okra (*Abelmoschus esculentus* L. Moench), during midcrop stage, Zn concentrations were ~60 to 70% higher in plants treated with *Azotobacter* sp. and a cyanobacterium (*Calothrix*), compared to un-inoculated controls (Manjunath et al. 2016). Indeed, in the Microbox®-mediated experimental setup, discussed in **chapter 4**, exposure to FZB24 BVCs caused a 3-fold increase in Zn and Fe uptake in potato when compared to controls (S. O' Neill, personal communication, December 3, 2020).

The role of BVCs is further discussed in **chapter 2** and examines their use in both basic and applied plant-microbe research. Not only are bacterial volatiles important but microbial volatiles as a whole play important roles in this area of research, to address this fungal volatiles are also examined along with the methodologies employed to detect biogenic volatiles in general. Over 1000 BVCs have been identified to date (Sharifi and Ryu, 2018) and many of these have been shown to both induce ISR and inhibit the growth of phytopathogens. The most well-studied BVCs are arguably acetoin and 2,3-butanediol which were first described in Ryu et al. (2003) and Ryu et al. (2004) and possess both plant growth-promotion through cytokinin signalling and ISR-inducing activity. These BVCs have also been identified as part of work discussed in **chapter 4.** Not only can BVCs have growth-promoting effects on plants, but they can also have growth-inhibiting effects too. Hydrogen cyanide is a volatile compound which some bacteria use to competitively inhibit the growth of other bacteria, this can be helpful

as an indirect PGP activity when *Pseudomonas fluorescens* CHA0 was shown to inhibit the development of *Thielaviopsis basicola*, which causes black root rot in tobacco plants, through the production of HCN (Bailly and Weisskopf 2017) but concentrations that are too high can kill the plant as deleterious effects on growth were observed when *A. thaliana* was exposed to HCN, with a four-fold reduction in growth following exposure to 1 µmol HCN (Blom et al. 2011a). Therefore, careful consideration needs to be taken when selecting PGPR for growth enhancement, as under certain conditions this may not always occur. Tieing back in with the message of **chapter 1**, **chapter 2** also addresses the use of volatiles in biofortification strategies, as it was observed that under normal growth conditions, *B. subtilis* GB03 volatiles triggered a rise in the mRNA levels of Fe-deficiency-induced transcription factor 1 (*FIT1*), as well as two of its downstream targets, ferric reductase *FRO2* and the iron transporter gene *IRT1*. However, in *Arabidopsis fit1-2* knockout mutants, volatile-induced increases in iron assimilation and photosynthetic efficiency are impaired, suggesting that volatile-induced iron assimilation is mediated by FIT1 (Zhang et al. 2009). Further highlighting the multifaceted reach of VOCs in sustainable agriculture and food security as a whole.

Mitigating the ecological effect of synthetic plant protection products (PPP) is now enshrined in law within the European Union, of which Ireland is a member, under regulation ((EC) No 1107/2009 - plant protection products)[1], Ireland enacts this law nationally through (S.I. No. 155/2012 - European Communities (Sustainable Use of

Pesticides)[2] and (S.I. No. 159/2012 - European Communities (Plant Protection Products)[3]. A number of studies are investigating the use of integrated pest management (IPM) in order to address sustainable solutions to current and future problems in agriculture (Iriti and Vitalini, 2020) and this is being investigated in a number of different crops under a wider umbrella of sustainable and more ecologically-friendly approaches in grapevine (Pertot et al. 2017), cereals (Sarrocco et al. 2019), potato (Velivelli et al. 2014) and rice, where for example co-application of *Bacillus subtilis* strain MBI600 with a commercial fungicide 'azoxystrobin', at half the recommended rate improved the efficacy of *B. subtilis* MBI600, and over a period of four years reduced sheath blight severity to a level similar to that of azoxystrobin applied at the full rate in all four years. The synergistic effect of the combined treatment increased grain yield by 14 to 19% similar to that of the fungicide applied at the maximum recommended dose in three of the four years (Zhou et al. 2020), demonstrating that PGPR can be central to the march towards more sustainable agricultural production for the future.

The debate between conventional versus organic agriculture and which approach is more optimal to plant and human health has intensified over recent years and forms the basis of our rationale for soil bacterial isolations in **chapter 3**. A target has been set by the EU to reach 25% organic production across the union by 2030, in 2018 the EU average organic share of the total ultilised agricultural area was 7.5%, and from the period 2012-2018 there was a 33.7% increase in total organic area

(fully converted and under conversion)[4]. From an Irish context just 1.4% of total agricultural land area was under organic production in 2018[5]. While organic production is increasing across the EU it is difficult to see a target of 25% total production within nine years, especially as the increased price for organic food is already a major barrier. The key findings from an Irish Food Board (Bord Bia) report on consumer attitudes towards organic food in 2020 state that 47% of Irish consumers are willing to pay more for organic produce, but that the predicted premium is probably just 10% extra and for two out of every three consumers, locally grown produce is more attractive than an organic option[6]. These findings show that, while becoming more widespread, organic production still has some significant hurdles to overcome, and with economic uncertainty growing amid the COVID-19 pandemic, organic production faces further challenges as a result of this and conventional production will most likely remain the mainstay well into the foreseeable future. To this end both organic and conventional sites were selected from which to obtain potential PGPR from potato rhizospheric soils across the Southern coast of Ireland, discussed in **chapter 3.** There was a considerable amount of overlap in isolate identity among 21 bacterial isolates obtained from both cropping regimes, which included *Achromobacter marplatensis*, *Pseudomonas helmanticensis* and *Bacillus mycoides* all of which possessed PGP traits, *P. helmanticensis* possessed all five PGP traits that were screened for, showing that regardless of the cropping regime PGPR are present and can live under both regimes. It is well reported

that soil microbes can cope with and even degrade synthetic PPPs giving an adaptive advantage to life under conventional cropping regimes (Kumar et al. 2018; Jiang et al. 2019) and recently it was reported that Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes and Bacteroidetes were identified as the dominant phyla present in both organic, and conventional, farming systems (Armalyté et al. 2019). The PGP activities screened for in this study are important for sustainable agriculture particularly P solubilisation and siderophore production discussed in **chapter 3,** but particular attention is focussed on the potential of VOCs for sustainable agriculture. Bacterial volatile compound (BVC)-mediated growth inhibition of *R. solani* was prevalent among the 21 isolates tested with levels ranging from 16.92 % to 47.34 % inhibition. The fact that VOCs can act as both direct and indirect PGP agents and that they can also be applied as pure compounds in the absence of microbes makes them ideal potential candidates for alternatives to synthetic PPPs in sustainable agricultural systems and can also address SDGs such as Zero Hunger, Responsible Consumption and Production, Climate Action and Life on Land.

Through the Microbox® system described in **chapter 4** it was determined that all five of the selected isolates from Irish potato soils as well as one commercial *Bacillus* isolate were all capable of BVC-mediated plant growth promotion and their BVC profiles were determined in three different media types of varying carbon content using SPME-GC/MS, as media type is known to have an effect on BVC emission profiles (Blom et al. 2011). The strongest effects were seen in

P. azotoformans BRP14 and *B. mycoides* LAM7 with a 2.57- and 2.69-fold increase in dry weight compared to that of the control with axenic media treatment respectively, with each isolate being from a commercial and organic potato crop respectively. The commercial isolate *B. amyloliquefaciens* FZB24® has been well characterised as a PGP agent which is capable of BVC-mediated biocontrol activity (Elanchezhiyan et al. 2018; Nguyen et al. 2019; Bahramisharif and Rose, 2019), and *Serratia* isolates are well-reported in BVC-related biocontrol literature (Soenens and Imperial, 2019; Dandurishvili et al. 2011; Kai et al. 2010) therefore, we selected FZB24 and LAC2 as BVC-emitting isolates for co-cultivation with potato in our experimental setup followed by MACE-seq transcriptomic analysis to identify key differentially expressed genes post-BVC exposure.

In **chapter 4**, upregulated genes were identified from the PGSC genome ($Log2FC > 2.0$, $p < 0.05$) and largely comprised those involved in photosynthetic processes, as described in similar previous studies (Sharifi and Ryu, 2018), as well as an immune response attenuator, phytosulfokine peptide (Igarishi et al. 2012). Unexpectedly, it was observed that downregulated genes involved many related to defence and stress responses in plants, in general it has been reported in the literature that defence genes are upregulated following the exposure to BVCs from beneficial microbes through the induction of ISR (Ryu et al. 2004; Sharifi and Ryu, 2016; Garbeva and Weisskopf, 2020). However, more recent literature has shown that this may not always be the case, Lee et al. (2019) demonstrated that a VOC from a beneficial

Trichoderma, 1-decene, applied as a pure chemical standard could increase plant fresh weight and chlorophyll content and downregulate defence- and stress-related genes in *Arabidopsis* (Lee et al. 2019). When *Trichoderma* comes into direct physical contact with *Arabidopsis,* salicylic acid (SA)- and jasmonic acid (JA)-related defence genes are downregulated, thus allowing *Trichoderma* to colonize plant roots (Morán-Diez et al. 2012). Downregulation of some stress response, abiotic and biotic stimuli genes are also downregulated when PGP bacteria come into direct contact with *Arabidopsis* (Poupin et al. 2013). Lee et al. propose that *Trichoderma* may use PGP VOCs to downregulate plant defence and stress responses prior to physical contact with the plant to allow a more effective initial colonisation. I propose that the same phenomena can occur with bacteria as it can with fungi and the molecular evidence supports this hypothesis. From an evolutionary perspective the transcriptomic analysis may make sense, as upregulating the attenuation of plant defence can have beneficial effects on plant growth, as there is a cost/benefit trade-off between defence and growth (Igarashi et al. 2012), upregulating genes related to photosynthetic processes can increase plant growth and release of root exudates and downregulation of defence and stress responses can also be tied into this observation and may benefit PGPR which live very close to the host plant and metabolise carbon released in root exudates from a healthy plant or, eventually make their way into the plant endosphere to live as endosymbionts.

The application of PGPR and their volatiles to biotechnology is discussed in **chapter 5**, in relation to *Agrobacterium tumefaciens*-mediated transformation (AMT) for agricultural applications and integration into a wider IPM strategy. For decades research groups have been attempting to increase transformation rates of *Agrobacterium* by manipulating the abiotic and biotic factors involved in the bacterial-host interaction, ranging from pH, temperature, photoperiod, phytohormone levels, binary plasmids, *Agrobacterium* virulence gene expression and host defence gene expression to name but a few (Nonaka et al. 2019; Anand et al. 2018; Chen et al. 2018; Hada et al. 2018; Rai et al. 2012). In a Microbox® experimental setup we could achieve up to 6- and 8-fold post-BVC exposure increases in transformation via *A. tumefaciens* strain EHA105 harbouring pCAMBIA 1305.2, where whole potato microplants were exposed to BVCs for a period of four weeks. More modest increases were observed in *A. tumefaciens* strain AGL1 using the same experimental procedure. A study by Plyuta et al. (2016) investigated the effect of the VOCs 2-nonanone, 2-heptanone and 2-undecanone and found that they could effectively inhibit the formation of *Agrobacterium* biofilms in an I-plate system (Plyuta et al. 2016). The LAC2 isolate from this study is also capable of emitting these BVCs, discussed in **chapter 4**. It is interesting to note that pre-treatment of plants with these BVCs allowed increased transformation rates with *Agrobacterium* post plant BVC-exposure. It may be the case, from an evolutionary perspective, that plants recognise "beneficial" BVCs that would usually inhibit the

growth of plant phytopathogens such as *Agrobacterium* and as a result, downregulate their defence and stress gene expression.

The inhibition of *Agrobacterium* growth and as a result, genetic transformation, by salicylic acid (SA)-mediated defence signalling is well reported (Wei et al. 2019; Rosas-Díaz et al. 2017). A number of genes associated with the SA-defence are downregulated in this study, discussed in **chapter 4** such as *EDS1*, *SGT1*, and *PEN1*. Special attention can be paid to *PEN1* as it aids penetration resistance to the cell, preventing invasion of the host by phytopathogens and downregulation of this gene may aid invasion by *Agrobacterium*, in particular *EDS1* is known to be one of the key genes involved in the feedback loop of SA-mediated R gene activation (Shah 2003) and downregulation of this gene may help to enhance AMT, as can be seen in this study. Societal challenges to GM technology still exist, especially in the case of transgenesis, however cisgenesis may provide a more acceptable alternative as it has been shown that stacking of multiple potato R genes in the potato cultivar 'Desiree' via AMT and subsequent marker-free selection could induce durable resistance to the fungal phytopathogen *Phyphthora infestans* in field trials conducted in the Netherlands and Ireland from the period 2013-2014, and 2015 in Ireland only, showed increased resistance to *P. infestans* compared to controls and resulted in an 80% decrease in fungicide applications. Cisgenesis was found to be more publically acceptable and fields where the crop trials were taking place were made accessible to the public on

open days to allow greater transparency (Kessel et al. 2018; Haverkort et al. 2016)

The future of agriculture is one of sustainability as there is no other viable option to protect both the planet and food security in the face of global climate change and an exponential human population increase. PGPR and indeed many other soil microbes such as PGPF represent a colossal untapped resource both as direct amendments to agricultural crop growth systems and sources of novel metabolites with agronomical interest. As organic cultivation of crops has become more popular worldwide it has more than doubled the amount of land used for this type of agriculture from 31, 815 hectares to 69,217 in the period 2007-2017 and in the same period, emissions from agriculture worldwide have increased from 5.01 to 5.41 gigatonnes (FAOSTAT, 2020). The trend of both pesticide use and N_2O output from synthetic fertiliser use in Ireland for example has increased over the past number decades (Fig. 1 and Fig. 2), N_2O acts as both a greenhouse gas and ozone depletion agent (Ravishankara et al. 2009), and therefore its emission, especially from agriculture, needs to be minimised.

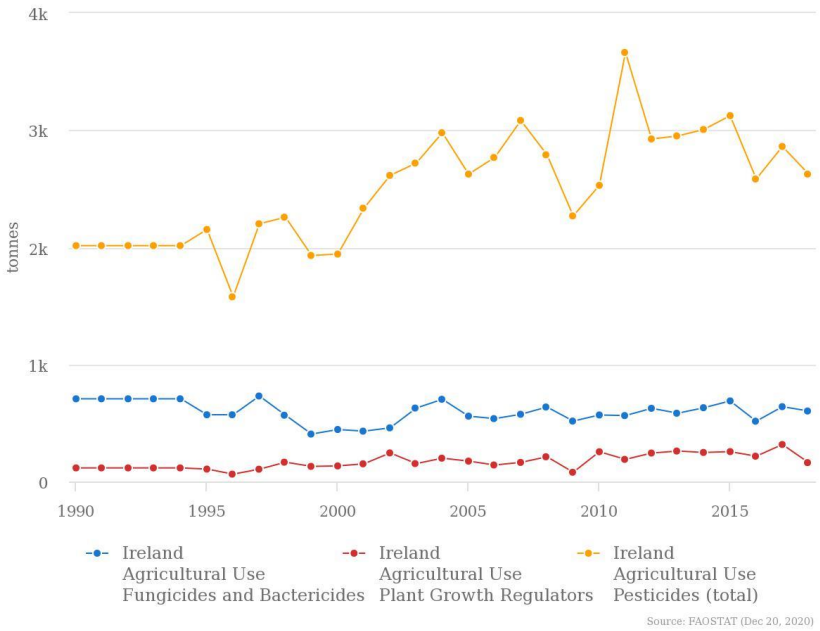


Fig. 1. The agricultural use of fungicides and bactericides, plant growth regulators and pesticides in Ireland over the period 1990-2018. Source: FAOSTAT, 2020.

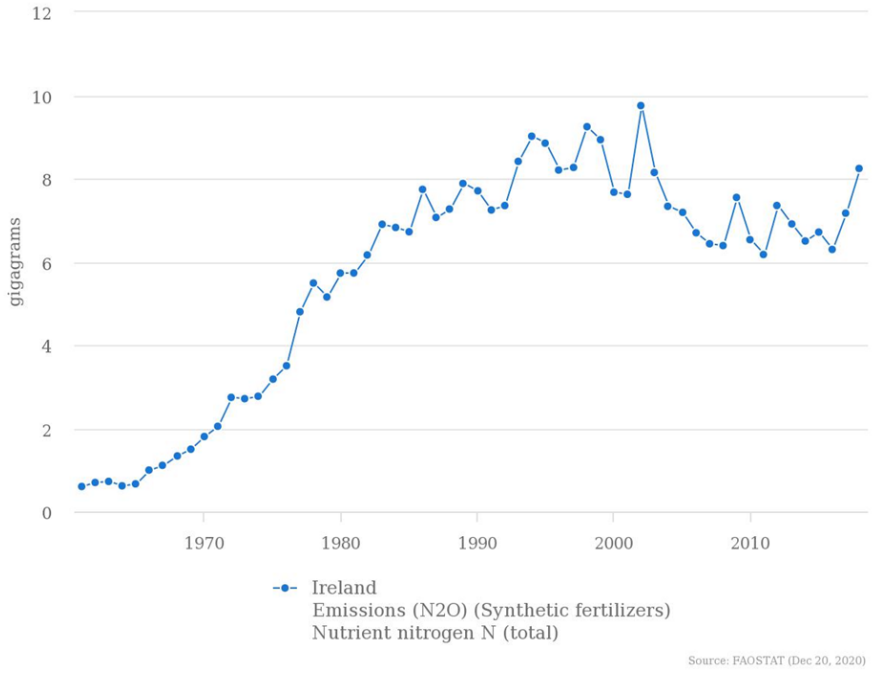


Fig.2. Ireland's N_2O emissions from synthetic fertiliser use for the period 1961-2018. Source: FAOSTAT, 2020. N_2O acts as both a greenhouse gas and ozone depletion agent.

Not only can PGPR help both conventional and organic agriculture but they can also play a part in finding more molecular biotechnological solutions to sustainable food production. Emission of some BVCs can dampen a plants immune responses thus allowing more efficient use of AMT to deliver fast, effective solutions either through rudimentary AMT-genetic transformation or through delivering new age gene-editing technologies such as CRISPR/Cas. The investigation of VOCs for sustainable agricultural solutions has rapidly gained traction over the past number of years (Garbeva and Weisskopf, 2020; Heenan-Daly et al. 2019) and while this technology is proving to be very promising a number of key questions remain, for example, which VOCs are defence inducers and which are defence repressors? How do in vitro experiments in controlled lab settings reflect what happens in the open field? How do VOCs interact with other biotic and abiotic players in agricultural settings? What is the right time to add the right VOC at different growth stages of different crops? Do all crop plants perceive VOCs in the same way? Will PGPR placed in field settings as BVC-emitters maintain a constant output over time? What molecular systems do plants use to perceive VOCs? With regard to the last question, our current understanding of the molecular mechanism(s) for plant sensing of VOCs is poor, research in the area has focussed mainly on the physiological response of plants and the ecological significance of VOCs. A recent study has shed light on plant perception on VOCs demonstrating that TOPLESS-like proteins act as transcriptional co-repressors that bind volatile caryophyllene analogues and, unlike

animals which use membrane-bound receptors to detect odours, plants, in this case tobacco, use a transcriptional co-repressor for both sensing and mediating the response to odours (Nagashima et al. 2019).

There is untold potential in the use of plant growth-promoting microbes for sustainable agriculture and humanity's future and indeed that of the planet as a whole can greatly benefit from both the continued research in this area and its direct applications in an ever-changing and ecologically uncertain future.

Concluding Remarks:

Findings from this study can greatly enhance sustainablilty in agriculture through the use of microbes and their metabolites and can help to meet the goals of a number of SDGs such as Zero Hunger, Good Health and Well-Being, Sustainable Cities and Communities, Responsible Consumption and Production, Climate Action and Life on Land. This study has provided greater insight into the complex communication strategies and the volatile-mediated 'language' microbes use to interact with plants and the wider ecosystem as a whole. The key outputs from this study are that Irish potato soils, both organic and conventional, harbour rhizobacteria that have PGP potential, BVCs from these bacteria have profound implications for plant growth and gene expression and can have organic, conventional and biotechnological applications. BVC blends may not always induce defence/stress responses in plants and instead, can downregulate these

responses, this can depend on a number of factors such as the BVC-emitting isolate, culture media of the isolate, exposure time to BVCs, or the experimental setup to mediate BVC-plant interactions, therefore, the multifactorial implications of this area of research still pose many questions left to be answered in future studies.

Footnotes:

[1]https://osha.europa.eu/en/legislation/directives/regulation-ec-no-1107-2009-plant-protection-products

[2] http://www.irishstatutebook.ie/eli/2012/si/155/made/en/print

[3] http://www.irishstatutebook.ie/eli/2012/si/159/made/en/print

[4]https://ec.europa.eu/eurostat/statistics-explained/index.php/Organic_farming_statistics#Organic_production

[5]https://www.cso.ie/en/releasesandpublications/ep/p-eii/environmentalindicatorsireland2020/landuse/

[6]https://www.bordbia.ie/globalassets/bordbia2020/industry/insights/new-publications/2020_organicresearch_rpt.pdf

References:

Anand A, Bass SH, Wu E, Wang N, McBride KE, Annaluru N, Miller M, Hua M, Jones TJ (2018) An improved ternary vector system for *Agrobacterium*-mediated rapid maize transformation. Plant Mol Biol 97:187-200

Armalytė J, Skerniškytė J, Bakienė E, Krasauskas R, Šiugždinienė R, Kareivienė V, Kerzienė S, Klimienė I, Sužiedėlienė E, Ružauskas M (2019) Microbial Diversity and Antimicrobial Resistance Profile in Microbiota From Soils of Conventional and Organic Farming Systems. Front Microbiol 10:892

Bahramisharif A, Rose LE (2019) Efficacy of biological agents and compost on growth and resistance of tomatoes to late blight. Planta 249:799-813

Bailly A, Weisskopf L (2017) Mining the volatilomes of plant-associated microbiota for new biocontrol solutions. Front Microbiol 8:1638

Blom D, Fabbri C, Connor EC, Schiestl FP, Klauser DR, Boller T, Eberl L, Weisskopf L (2011) Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. Environ Microbiol 13:3047-3058

Chen L, Cai Y, Liu X, Yao W, Guo C, Sun S, Wu C, Jiang B, Han T, Hou W (2018) Improvement of soybean *Agrobacterium*-mediated transformation efficiency by adding glutamine and asparagine into the culture media. Int J Mol Sci 19:3039

Dandurishvili N, Toklikishvili N, Ovadis M, Eliashvili P, Giorgobiani N, Keshelava R, Tediashvili M, Vainstein A, Khmel I, Szegedi E, Chernin L (2011) Broad-range antagonistic rhizobacteria *Pseudomonas fluorescens* and *Serratia plymuthica* suppress *Agrobacterium* crown gall tumours on tomato plants. J Appl Microbiol 110:341-352

Elanchezhiyan K, Keerthana U, Nagendran K, Prabhukarthikeyan SR, Prabakar K, Raguchander T, Karthikeyan G (2018) Multifaceted benefits of *Bacillus amyloliquefaciens* strain FBZ24 in the management of wilt disease in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Mol Plant Path 103:92-101

FAOSTAT (2020) Food and Agriculture Organization of the United Nations, statistics division, Viale delle Terme di Caracalla 00153 Rome, Italy

Fellmann T, Witzke P, Weiss F, Van Doorslaer B, Drabik D, Huck I, Salputra G, Jansson T, Leip A (2018) Major challenges of integrating agriculture into climate change mitigation policy frameworks. Mitigation and adaptation strategies for global change. 23:451-468.

Garbeva P, Weisskopf L (2020) Airborne medicine: bacterial volatiles and their influence on plant health. New Phytol 226:32-43

Hada A, Krishnan V, Jaabir MM, Kumari A, Jolly M, Praveen S, Sachdev A (2018) Improved *Agrobacterium tumefaciens*-mediated transformation of soybean [*Glycine max* (L.) Merr.] following optimization of culture conditions and mechanical techniques. In Vitro Cell Dev Biol Plant 54:672-688

Haverkort AJ, Boonekamp PM, Hutten R, Jacobsen E, Lotz LA, Kessel GJ, Vossen JH, Visser RG (2016) Durable late blight resistance in potato through dynamic varieties obtained by cisgenesis: scientific and societal advances in the DuRPh project. Potato Res 59:35-66

IPCC, 2021: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Masson-Delmotte, V., P. Zhai, A. Pirani, S.L. Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M.I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J.B.R. Matthews, T.K. Maycock, T. Waterfield, O. Yelekçi, R. Yu, and B. Zhou (eds.)]. Cambridge University Press. In Press.

IFIC (2020) International Food Information Council *2020 Food & Health Survey* https://foodinsight.org/2020-food-and-health-survey/ International Food Information Council, 900 19th St NW, Washington, DC 20006, USA

Iriti M, Vitalini S (2020) Sustainable Crop Protection, Global Climate Change, Food Security and Safety—Plant Immunity at the Crossroads. Vaccines 8:42

Kai M, Crespo E, Cristescu SM, Harren FJ, Francke W, Piechulla B (2010) *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. Appl Microbiol Biotech 88:965-976

Kessel GJ, Mullins E, Evenhuis A, Stellingwerf J, Cortes VO, Phelan S, van den Bosch T, Förch MG, Goedhart P, van der Voet H, Lotz LA (2018) Development and validation of IPM strategies for the cultivation of cisgenically modified late blight resistant potato. Eur J Agron 96:146-155

Kumar S, Kaushik G, Dar MA, Nimesh S, Lopez-Chuken UJ, Villarreal-Chiu JF (2018) Microbial degradation of organophosphate pesticides: a review. Pedosphere 28:190-208

Morán-Diez E, Rubio B, Domínguez S, Hermosa R, Monte E, Nicolás C (2012) Transcriptomic response of *Arabidopsis thaliana* after 24 h incubation with the biocontrol fungus *Trichoderma harzianum*. J Plant Physiol 169:614-20

Nonaka S, Someya T, Kadota Y, Nakamura K, Ezura H. (2019) Super-Agrobacterium ver. 4: improving the transformation frequencies and

genetic engineering possibilities for crop plants. Front Plant Sci 10:1204

Pertot I, Caffi T, Rossi V, Mugnai L, Hoffmann C, Grando MS, Gary C, Lafond D, Duso C, Thiery D, Mazzoni V (2017) A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. Crop Prot 97:70-84

Poupin MJ, Timmermann T, Vega A, Zuñiga A, González B (2013) Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS One 8:e69435

Jiang B, Zhang N, Xing Y, Lian L, Chen Y, Zhang D, Li G, Sun G, Song Y (2019) Microbial degradation of organophosphorus pesticides: Novel degraders, kinetics, functional genes, and genotoxicity assessment. Environ Sci Pollut Res 26:21668-21681

Manjunath M, Kanchan A, Ranjan K, Venkatachalam S, Prasanna R, Ramakrishnan B, Hossain F, Nain L, Shivay YS, Rai AB, Singh B (2016) Beneficial cyanobacteria and eubacteria synergistically enhance bioavailability of soil nutrients and yield of okra. Heliyon 2:e00066

Nagashima A, Higaki T, Koeduka T, Ishigami K, Hosokawa S, Watanabe H, Matsui K, Hasezawa S, Touhara K (2019) Transcriptional regulators involved in responses to volatile organic compounds in plants. J Biol Chem 294:2256-2266

Nguyen ML, Glaes J, Spaepen S, Bodson B, du Jardin P, Delaplace P (2019) Biostimulant effects of *Bacillus* strains on wheat from in vitro towards field conditions are modulated by nitrogen supply. J Soil Sci Plant Nutr 182:325-334

Rai GK, Rai NP, Kumar S, Yadav A, Rathaur S, Singh M (2012) Effects of explant age, germination medium, pre-culture parameters, inoculation medium, pH, washing medium, and selection regime on *Agrobacterium*-mediated transformation of tomato. In Vitro Cell Dev Biol Plant 48:565-578

Rana A, Kabi SR, Verma S, Adak A, Madan P, Shivay YS, Prasanna R and Nain L (2015) Prospecting plant growth promoting bacteria and cyanobacteria as options for enrichment of macro- and micronutrients in grains in rice-wheat cropping sequence. Cogent Food Agric 1: 1037379

Ravishankara AR, Daniel JS, Portmann RW (2009) Nitrous oxide (N2O): the dominant ozone-depleting substance emitted in the 21st century. Science 326:123-125

Rosas-Díaz T, Cana-Quijada P, Amorim-Silva V, Botella MA, Lozano-Durán R, Bejarano ER (2017) *Arabidopsis* NahG plants as a suitable and efficient system for transient expression using *Agrobacterium tumefaciens*. Mol Plant 10:353-356

Ryu C-M, Farag MA, Hu C-H, Reddy MS, Wei H-X, Paré PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci 100:4927–4932

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017-1026

Sarrocco S, Mauro A, Battilani P (2019) Use of competitive filamentous fungi as an alternative approach for mycotoxin risk reduction in staple cereals: state of art and future perspectives. Toxins 11:701

Shah J (2003) The salicylic acid loop in plant defense. Curr Op Plant Biol 6:365-371

Sharifi R, Ryu CM (2016) Are bacterial volatile compounds poisonous odors to a fungal pathogen *Botrytis cinerea*, alarm signals to *Arabidopsis* seedlings for eliciting induced resistance, or both?. Front Microbiol 7:196

Sharifi R, Ryu CM (2018) Revisiting bacterial volatile-mediated plant growth promotion: lessons from the past and objectives for the future. Ann Bot 122:349-358

Soenens A, Imperial J (2019) Biocontrol capabilities of the genus *Serratia*. Phytochem Rev 7:1-11

Wang YC, Yu M, Shih PY, Wu HY, Lai EM (2018) Stable pH suppresses defense signaling and is the key to enhance *agrobacterium*-mediated transient expression in *Arabidopsis* seedlings. Sci Rep 8:1-9

Wei H, Movahedi A, Xu C, Sun W, Li L, Li D, Zhuge Q (2019) Characterization, expression profiling, and functional analysis of a *Populus trichocarpa* defensin gene and its potential as an anti-*Agrobacterium* rooting medium additive. Sci Rep 9:1-7

Zhang H, Sun Y, Xie X, Kim MS, Dowd SE, Paré PW (2009) A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. Plant J 58:568–577

Zhou XG, Kumar KV, Zhou LW, Reddy MS, Kloepper JW (2021) Combined Use of PGPRs and Reduced Rates of Azoxystrobin to Improve Management of Sheath Blight of Rice. Plant Dis 105:1034-1041.