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Effects of strategic tillage and plant hormone treatments on wheat-associated microbial communities — Source link

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Effects of strategic tillage and plant hormone treatments on wheat-associated microbial communities

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Masters of Engineering (Food Quality and Safety)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

School of Agriculture and Food Sciences

Abstract

The main aim of this thesis was to characterise microbial properties of microbiomes associated with wheat (*Triticum aestivum*) soils and wheat plants. In particular my objective was to better understand the effects on microbial communities following strategic tillage (ST) in wheat field soils and the activation of plant defence pathways in wheat plants. Throughout the thesis, multiple culture-independent methods, especially next-generation sequencing of 16S rRNA gene were used to profile soil microbial communities. Over half of the arable land in the northern grains region of Australia is managed using no-till (NT), a farming method which has improved crop yields and soil quality while reducing the input and labour costs. However, in recent years, concerns have arisen among farmers over the weed control in continuous NT systems. Strategic tillage has been touted as a potential solution, in particular for the severe weed infestations of long-term NT. Nevertheless, there is little information on the influence of ST on the microbial properties of Australian NT soils. In the present study, ST applications and soil sampling were performed in Moonie, Moree and Condamine during the fallow period in eastern Australia. These sites were chosen based on their long history of repeated wheat cultivation under NT farming practices and their different soil types. Overall, results show that in the Moonie trial on a Calcisol, one-time ST with either chisel or offset disc did not significantly influence the composition of soil bacterial communities when measured 13 months after tillage. However, relative to the NT, chisel tillage led to significant increases in microbial biomass carbon (MBC), abundances of Alphaproteobacteria, Bacteroidetes and Firmicutes as well as the utilisation of D+cellubiose and mannitol at 0-10 cm depth. In the Moree trial on a grey Vertosol, ST with different timing and implement demonstrated great potential in weed control and did not influence wheat yield and soil physicochemical and biological properties in the short-term. In the Condamine site on a Solonetz soil, one- or two-time chisel tillage did not influence soil MBC, total microbial enzymatic activity (MEA) or utilisation of C substrates. Likewise, ST did not change the soil microbial community structure and the abundance of genes encoding enzymes involved in key steps of C and N reactions. However, one-time chisel increased relative abundance of Acidobacteria RB41 and Acidobacteria iii1-15, and two-time chisel slightly increased the average C utilisation, at 10-20 cm depth. Overall, this thesis suggests that ST does not cause major impacts on soil properties of long-term NT and could be potentially used to address the long-term NT-associated issues without impacting overall soil properties.

Recent reports demonstrate the importance of microbiomes associated with plants and their soil they are cultivated in. Beneficial microbes can significantly increase crop yields and provide biocontrol functions against plant pathogens, but prior to this study little was known how wheat plants and their physiological pathways influence associated microbiomes *in planta* and

surrounding soil. We hypothesised that microbiomes in soils with a long history of repeated wheat cultivation and NT practice harbour microbes that are well-adapted to wheat plants. Therefore, we cultivated wheat using the wheat field soils collected from Moonie and Condamine and tested the effects of the activation of plant signalling pathways on the wheat microbiomes. Jasmonic acid (JA) and salicylic acid (SA) pathways were chosen based on their key roles in plant defence against biotrophic and necrotrophic phytopathogens, respectively. Seventy-two hours after methyl jasmonate (MeJA) treatment on wheat shoots, the composition of microbial communities in endophytic roots was altered. Decreased microbial diversity was observed in endophytic roots. In contrast, I found no evidence that microbial communities in endophytic shoots or rhizosphere were affected by MeJA treatment. Using Moonie and Condamine soils, I tested the effect of the activation of SA signalling on the composition and functions of wheat rhizosphere microbial communities. Seventy-two hours after SA treatment, the enhanced SA signalling marginally changed the composition of rhizosphere microbial communities in the Solonetz but not in the Calcisol. In particular, SA signalling triggered a significant decrease in *Sphingobacteria* and the Archaea member *Nitrososphaera,* but only in the Solonetz, not in the Calcisol rhizosphere. In addition, the copy numbers of *arch-amoA, nifH*, *amoA* and *nosZ* genes were reduced in the Solonetz rhizosphere by SA treatment. Taken together, it appears that JA and SA signalling pathways influence wheat-associated rhizosphere and endophytic microbial communities differentially. Wheat plants with activated JA-dependent defence may harbour bacterial communities of lower diversity in the root endosphere while an enhanced level of SA signalling may lead to a decrease of microbial components involved in N cycling in rhizosphere soil. Future studies may focus on how wheat-associated microbiomes may contribute to higher yields and improved crop resilience. This could be achieved through detailed characterisation of individual plant-microbe interactions, by engineering wheat-optimised microbiomes or by breeding wheat cultivars with improved microbiome interactions.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

1. **Hongwei Liu**, Lilia C. Carvalhais, Kemal Kazan, Peer M. Schenk (2016) "Development of marker genes for jasmonic acid signaling pathway in shoots and roots of wheat." ***Plant Signalling and Behavior*** 11(5): e1176654.
2. Mark Crawford, Vivian Rincon-Florez, Anna Balzer, Lilia C. Carvalhais, Yash Dang, **Hongwei Liu**, Peer Schenk (2015) "Changes in the soil quality attributes of continuous no-till farming systems following a strategic tillage." ***Soil Research*** 53(3):263-273.
3. **Hongwei Liu**, Lilia C. Carvalhais, Vivian Rincon-Florez, Mark Crawford, Yash P. Dang, Paul G. Dennis, Peer M. Schenk (2016) "One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol." ***Soil and Tillage Research*** 158(5):91-99.
4. **Hongwei Liu**, Mark Crawford, Lilia C. Carvalhais, Yash P. Dang, Paul G. Dennis, Peer M. Schenk (2016) "Strategic tillage on a Grey Vertosol after fifteen years of no-till management had no short-term impact on soil properties and agronomic productivity." ***Geoderma*** 267:146-155.
5. **Hongwei Liu**, Lilia C. Carvalhais, Mark Crawford, Yash P. Dang, Paul G. Dennis, Peer M. Schenk (2016) "Strategic tillage increased the relative abundance of Acidobacteria but did not impact on overall soil microbial properties of a 19-year no-till Solonetz." ***Biology and Fertility of Soils*** 52(7):1021-1035.

Book Chapter

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Conference abstracts

1. Rincon-Florez VA, Carvalhais LC, Dang YP, **Liu H**, Crawford MH, Bell MJ, Schenk PM (2015) Impact of strategic tillage on nitrogen cycle genes (*amoA* and *nifH*) in no-till systems in Queensland Australia. Ecology of soil microorganisms, Prague, 29 November-3 December (Poster).
2. **Liu H**, Mirzaee H, Carvalhais LC, Dennis PG, Schenk PM (2015) Activation of salicylic acid defence signaling pathway reduced Archaea abundance and genes involved in nitrogen and carbon cycling in wheat rhizosphere. Ecology of soil microorganisms, Prague, 29 November-3 December (Poster).
3. **Hongwei Liu**, Lilia Carvalhais, Paul Dennis, Peer Schenk (2015) Development of marker genes for jasmonic acid signaling pathway in shoots and roots of wheat. Tropical Agriculture conference, Brisbane, 16-18 November 2015 (poster).
4. **Hongwei Liu**, Vivian Rincon-Florez, Yash Dang, Mark Crawford, Anna Balzer, Mike Bell, Dalal Ram, Lillia Carvalhais, Peer Schenk (2014) Strategic tillage impacts on biological attributes of long term no-till soils in northern grain region of Australia. National soil science conference, Melbourne, 23-27 November (poster).

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Contributor	Statement of contribution
Author: Hongwei Liu (Candidate)	Designed experiments (60%) Performed experiments (100%) Data analysis and interpretation (50%) Wrote the paper (60%)
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Author: Vivian Rincon-Florez	Designed experiments (5%) Wrote and edited paper (5%)
Author: Mark Crawford	Designed experiments (10%) Wrote and edited paper (5%)
Author: Yash P. Dang	Designed experiments (10%)
Author: Paul G. Dennis	Data analysis and interpretation (50%) Wrote and edited paper (5%)
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Author: Peer M. Schenk	Designed experiments (10%) Data analysis and interpretation (15%) Wrote and edited paper (10%)

Contributions by others to the thesis

Bioinformatics of 16S rRNA Illumina sequencing, analysis of the microbial changes in soil and/or plant in chapter 2, 6 and 7 were greatly assisted by Dr Paul G. Dennis. Dr Lilia C. Carvalhais helped with the plant MeJA treatments, rhizosphere and wheat samplings as well as data interpretation of RT-PCR data. Soils for wheat cultivation were collected by Anna Balzer and Dr Yash P. Dang. Critical conceptions, experimental design, interpretation of research data was guided by Prof Peer Schenk and Dr Lilia C. Cavalhais.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

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Keywords

archaea, endophyte, jasmonate, microbial community, plant defence, salicylic acid, soil, soil nitrogen cycling, strategic tillage, wheat.

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ANZSRC code: 050303, Soil Biology 50%

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Fields of Research (FoR) Classification

FoR code: 0605, Microbiology, 70%

FoR code: 0701, Agriculture, Land and Farm Management, 20%

FoR code: 0602, Ecology, 10%

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List of Abbreviations

AGRF	Australian genome and research facility
amoA	Ammoniamonoxygenase subunit A gene
arch-amoA	Archaea ammoniamonooxygenase subunit A gene
ANOVA	Analysis of variance
AOA	Ammonia-oxidising archaea
AOB	Ammonia-oxidising bacteria
C	Carbon
CLPP	Community level physiological profile
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FAO	Food and agriculture organization of the United Nations
FDA	Fluorescein diacetate
Fe	Iron
GRDC	Grain research and development corporation
N	Nitrogen
narG	Nitrate reductase gene
nifH	Nitrogenase gene
nosZ	Nitrous oxide reductase gene
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
NT	No-till
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
Q-PCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SOC	Soil organic carbon
ST	Strategic tillage
T-RFLP	Terminal restriction fragment length polymorphism
NGS	Next generation sequencing
SA	Salicylic acid
OTU	Operational taxonomic unit

Chapter 1 Research aims and literature review

1 Research questions and research aims

1.1 Research questions

- Does strategic tillage (ST) exert negative impacts on soil microbial benefits accumulated by long-term NT practices in Australian wheat cropping systems?
- Does ST using different implements, frequency or timing make a difference regarding ST effects on soil microbial properties?
- What are the composition and diversity of wheat-associated microbial communities and how do they differ between bulk soil, rhizosphere soil and interior plant sections?
- Does the activation of the jasmonate (JA) defence signalling pathway influence wheat-associated microbial communities?
- Do the microbial communities in different wheat compartments respond to the activation of JA signalling defence pathway differentially?
- Does the activation of SA defence signalling pathway influence the wheat-associated microbial community structure?
- Does soil type influence the response of plant-associated microbial communities under activation of plant defence signalling pathways?

1.2 Research aims and hypothesis

- AIM 1: To examine the influence of ST using chisel and offset disc on the soil microbial properties of a long-term no-till (NT) Calcisol at Moonie, Queensland, Australia. I tested the hypothesis that one-time ST using a minimal inversion implement does not cause major impacts on the soil biological attributes of the long-term NT. This is covered in chapter 2 of this thesis.
- AIM 2: To identify the possible impacts of ST applied at different times in the fallow and using different types of implements on soil agronomic productivity, physicochemical and biological properties of a long-term NT Vertosol in Moree, New South Wales, Australia. I hypothesised that ST with different timing and implement type does not change soil properties and

agronomic productivity of the 19-year NT grey Vertosol in the short-term. This is covered in chapter 3 of this thesis.

- AIM 3: To examine the short-term effects of ST applied with different frequency on soil biological properties of the long-term NT Solonetz. I hypothesised that ST effects on the composition, microbial activity, and N and C cycling potentials of the microbial communities in the NT soil are small. This is covered in chapter 4 of this thesis.
- AIM 4: To examine the effects of an elevated JA signalling on the wheat associated microbial communities using the NT Solonetz soil collected from one tillage site in Condamine. I hypothesised that wheat-associated microbial communities are altered by the elevated JA signalling and the strongest change occurs in the endophytic root compartment of wheat seedlings because it links soil and plant influences. This is covered in chapter 5 of this thesis.
- AIM 5: To examine the effects of an elevated SA signalling on wheat associated microbial communities using both the Solonetz and Calcisol soil that were respectively collected from Moonie and Condamine. I hypothesised that the microbial communities in wheat rhizosphere are altered by an elevated SA signalling pathway, and the changes are also influenced by the different soils used for wheat cultivation. This aspect is covered in Chapter 6 of this thesis.

2 Literature review

Soil bacteria, fungi, archaea and protists constitute the most diverse living communities on Earth and provide essential ecosystem services to life on this planet. These microorganisms reside in soil and plant-associated niches including ectorhizosphere (or rhizosphere as often non-discriminately used), rhizoplane and endosphere (Fig. 1). Soil provides plants with nutrients, water and a matrix of anchorage and the rhizosphere is the immediate soil surrounding plant roots (Bais et al., 2006). The microbial communities in the rhizosphere greatly influence plant health and performance and themselves are influenced by root secretions (Berendsen et al. 2012). The endosphere inside plant tissues also harbours a large number of microbial cells (~10^4-10^8 g^{-1} roots) that do not cause visible symptoms on plants but have documented effects on plant growth and health (Bulgarelli et al. 2013). As the effects of strategic tillage and plant hormone signalling factors on either soil or wheat associated microbial communities (in rhizosphere and endosphere) were examined in this thesis research, soil and rhizosphere are reviewed in the first place with aspects to the microbial community structure and microbial properties. I separately reviewed multiple aspects of the plant endosphytic bacterial communities regarding their interactions with plants and a review manuscript is included in the appendix 1 of this thesis.

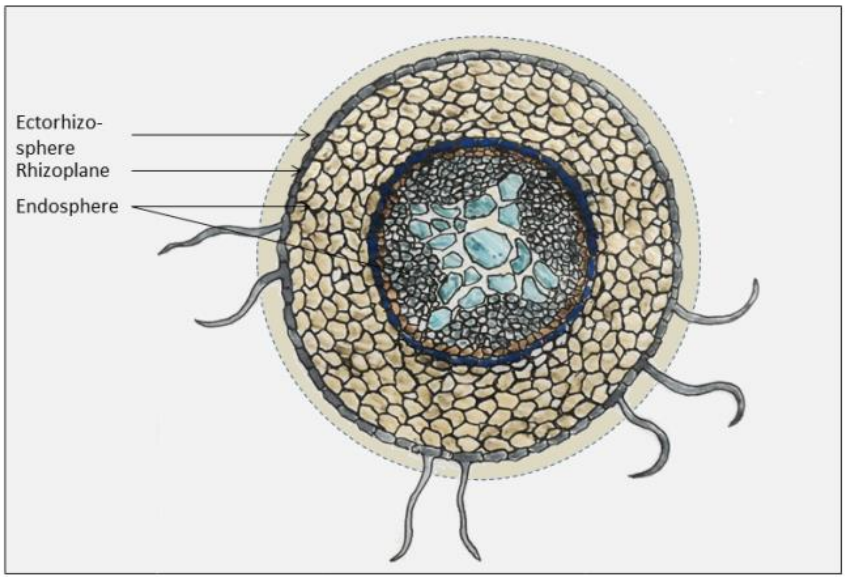

Fig. 1 Illustration of the different compartments of a plant root (shown as a cross section) that harbour diverse microorganisms.

2.1 Soil microbial communities

Soil microbial communities are tremendously diverse in both the taxonomic structure and biological functions. A single gram of soil contains up to 10^{10} microbial cells and tens of thousands of bacterial and archaeal species (Berendsen et al. 2012). These microbes have critical roles in soil nutrient cycling, soil formation and also influence plant health and performance. Many soil microbes, e.g., Archaea and Acidobacteria, are currently still difficult to culture. However, a recent report showed that up to 70% of soil microbes associated with *Arabidopsis* plant roots could be cultured by using a variety of different cultivation media (Bai et al. 2015). Whole soil analysis method may circumvent soil microbial culture and provide an alternative to study the profile and function of soil microbiomes. For instance, the Microresp™ is a commonly used method to determine substrate utilisation, also referred to as community level of physiological profile (CLPP) (Nannipieri et al. 2013). The newly emerging techniques of high-throughput sequencing (also known as next generation sequencing (NGS)) allow the profiling of the taxonomic and functional structures of soil microbial communities via analysis of phylogenetic marker genes (e.g., 16S ribosomal RNA and *nifH* gene) or the metagenome of a soil sample (Fierer et al. 2012).

While soil-borne pathogens, such as *Fusarium*, *Pythium* and *Phytophthora* ssp. often receive a lot of attention, the vast majority of soil microbes can be considered neutral (commensals) or beneficial to plants. It has been shown that plants grown in axenic soil may only produce half the

biomass of plants grown in the presence of soil microbes (Carvalhais et al. 2014). Beneficial soil microorganisms play a major role in plants for nutrient acquisition (e.g. by N fixation or P solubilisation) and disease suppression (e.g. by production of siderophores, antimicrobial compounds or anti-fungal chitinases). For this reason, plants release large amounts of organic carbon (sugars and organic acids) into the ectorhizosphere to recruit soil microbes that provide benefits to the plants. The direct addition of certain or mixed microorganisms (e.g. *Bacillus subtilis* or *Trichoderma*) to soil has also been practised with varying results to improve plant nutrition and/or disease resistance (Cao et al. 2011; Kavoo-Mwangi et al. 2013). There is mounting evidence that plants can selectively attract and maintain rhizosphere microbes by root exudates to gain benefits, but the chemical language and services from these types of soil microbiome manipulations are often still poorly understood or unknown, especially for commercial crop plants. The well-studied legume-rhizobia interactions provide a good example for a chemical language where specific compounds attract specific rhizobacteria (Fierer et al. 2007). It can be expected that similar common principles between compounds, attracted microbe and function can be established for other parts of the rhizosphere microbiome, although some of them will be less specific.

Among the soil microorganisms, bacteria are the most abudant. Proteobacteria (mainly the α-, β- and γ- subdivisions), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes and Firmicutes are normally the major bacterial phyla in soil (Janssen, 2006). Some bacterial groups have been documented with some outstanding traits. For instance, Actinobacteria play an important role in soil nutrient mobilisation and are among the most efficient groups in producing secondary metabolites that relate to plant disease suppression (Palaniyandi et al. 2013). Many Bacteroidetes are increasingly considered as specialists in degrading organic matters of high molecular weight such as cellulose, pectin and xylan (Thomas et al. 2011). The bacterial phyla Actinobacteria, Proteobacteria and Firmicutes harbour a diverse range of plant growth promoting bacteria (PGPB) such as *Pseudomonas fluorescens* WCS417, *Bacillus subtilis*, and N_2 fixing bacteria *Rhizobium* and *Bradyrhizobium* (Compant et al. 2005). It has also been revealed that bacterial taxa from Proteobacteria (e.g., Pseudomonadaceae, Burkholderiaceae, and Xanthomonadales), Actinobacteria and Firmicutes (e.g., Lactobacillaceae) may be associated with the suppression towards the fungal pathogen *Rhizoctonia solani* in the rhizosphere of sugar beet seedlings (Mendes et al. 2011).

2.2 Factors driving soil microbial communities

Soil microbial communities change in abundance, composition and diversity, which are generally driven by many interacting environmental factors. In natural ecosystems, soil types, climate, biotic interactions, plant species and diversity are among the determinant factors that shape the soil microbial community structure (Philippot et al. 2013). The richness and diversity of soil microbiota are highly dependent on the types of ecosystems and significantly correlate to soil pH (Fierer and Jackson 2006). Neutral soils may have the highest bacterial diversity while acidic soils have lower bacterial diversity (Fierer and Jackson 2006). Soil microbial communities have also been influenced by humans in many ways since the advent of agriculture (Matson et al. 1997). These include (1) soil amendments (additives to soil, such as fertilisers, pesticides, charcoal, manure or other organic matter sources) (Matson et al. 1997; O'Donnell et al. 2001). Soil organic C (SOC), for example, provides a source of energy and nutrients for soil microorganisms, and the addition of manure or fertilisers in soils may change SOC and therefore change the soil microbial activities and the community structure (O'Donnell et al. 2001); (2) crop rotation or mixed crop systems (e.g. legumes biofertilise the soil or the use of allelopathic plants) (Lupwayi et al. 1998); and (3) soil cultivation (Jackson et al. 2003). Tillage practices, in particular, may change soil moisture, soil aggregation and distribution of pore sizes, which thereby influence soil microorganism access to oxygen, water and nutrients (Ghimire et al. 2014). Overall, soil microorganisms are affected by many factors in natural and agricultural ecosystems. Due to the importance of soil microbes in agroecosystem, investigating the potential effects of soil management practices on the soil microbial diversity may contribute to the development of sustainable agriculture and address some environmental issues.

2.3 Soil-tillage systems

Tillage practices influence soil physicochemical and biological attributes in agro-ecosystems, which could be a principle factor influencing agronomic productivity and eco-sustainability (Bronick and Lal 2005). Worldwide tillage practices are generally categorised into conventional tillage, conservation tillage/reduced tillage and no-till (NT)/zero tillage (Unger 1990). Conventional tillage is also known as aggressive tillage that inverts soil and incorporates crop residues into subsoil with <15% left on soil surface (Daughtry et al. 2004). Adoption of conventional tillage may cause soil erosion, loss of soil moisture and organic matter, disturbance of soil structure and disruptions in soil biota (Dang et al. 2015). These conventional tillage-associated disadvantages reduce agronomic productivity and soil sustainability (Mathew et al. 2012). Conservation tillage retains at least 30% of crop residues on soil surface, which may contribute to a better water infiltration and reduces soil erosion relative to conventional tillage. No-tillage, also known as zero

tillage or direct drilling, refers to the practices of growing crops from year to year without tillage disturbances on soil (Ismail et al. 1994; Six et al. 2000; Thomas et al. 2007).

Australia has about 17 Mha of fields currently being under NT farming, accounting for 11.4% of the world NT (160 Mha) arable soils in 2014 (FAO 2014). In Queensland (QLD), Australia, NT represents approximately 50% of the total cropping area but the rate can be up to 85% in some regions of QLD (Thomas et al. 2007). No-tillage has tangible economic advantages over conventional tillage as it requires less labour and fuel consumption, and potentially increases crop yields (Dang et al. 2015). Environmentally, NT practices can maintain soil microbial biomass C and soil water, reduce the risk of soil erosion and largely protect biological diversity in the agroecosystem. Higher microbial activity, fungal and bacterial abundances are visually observed in NT systems than conventional tillage systems (Dang et al. 2015). In particular, NT may significantly improve crop performance and yields in low rainfall farming zones due to the increased soil water holding capacity (Blevins et al. 1971).

However, long-term NT managed soils may cause inconveniences for growers. These typical NT-related issues in Australian agriculture systems include nutrient accumulations on soil surface (0~5 cm), build-up of soil- and stubble-borne diseases and the prevalence of herbicide resistant weeds. These problems can lead to nutrient runoff, compromised soil quality and decreased agronomic productivity (Dang et al. 2015). Across Australian northern grains-growing regions (NGR), several dominant weed species have developed strong resistance to glyphosate, such as annual ryegrass (*Lolium rigidum*), barnyard grass (*Echinochloa colona*) and fleabane (*Conyza bonariensis*) (Dang et al. 2015). For controlling these herbicide-resistant weeds, more herbicide (e.g., glyphosate) usage is required, but this could cause a cost increase and more environmental impacts. Australian growers confronted with these issues claim that they cannot continue NT practices and that they must return to ploughing to solve weed problems. However, growers have the concerns that even one-time tillage event may undo the benefits accumulated by long-term NT practices.

Competitive crops, organic manure amendments, crop rotation and weed seeds gathering are management options but these may still not be sufficient to address NT-related issues in particular soil type (Kirkegaard et al. 2014). Tillage practices are generally effective in solving the weed issues in long-term NT managed soils and it has been used for this purpose in traditional agriculture since ancient times. Some forms of tillage overturn soils, bury weeds in soil. Overall, without sunlight and nutrients assimilation from soil, weeds are then killed by soil tillage. In Western

Australia, some growers have performed tillage once per ten years on moist soil to kill herbicide resistant weeds; simultaneously, lime was incorporated deep into soils during times of low risk of erosion (Kirkegaard et al. 2014). This is known as one-off use in conservation farming. Tillage in any form will inevitably change the soil physical and chemical properties and the habitats of soil biota. It is this change and the impact on productivity that needs to be assessed to fully understand the risks associated with tillage applications in NT fields. In the following section, effects of occasional tillage on soil microbial properties are reviewed.

2.4 Effects of occasional tillage on soil microbial properties of long-term NT soils

Any type of tillage may cause disturbance of soil structure, soil water and oxygen content, soil temperature and potentially influence soil physico-chemical and microbial properties. Strategic tillage is the opportunistic use of occasional tillage in an otherwise NT system to address specific biotic or abiotic stresses (Dang et al. 2015). It aims to avoid any major impacts on soils by using minimal inversion tillage implements at the right timing. Multiple previous studies carried out in countries other than Australia have shown inconsistent results regarding effects of one-time tillage on soil microbial properties of NT soils. Soil microbial biomass carbon (C) and nitrogen (N) as well as microbial enzyme activities of dehydrogenase, beta-glucosidase, and diphenol oxidase were decreased by one-time tillage with a mouldboard plough (MP) (López-Garrido et al. 2011; Melero et al. 2011). One-time MP applied to a NT clay loam soil also reduced root colonisation by arbuscular mycorrhizal fungi. However, some other studies reported no negative effect of one-time tillage on NT soils (Garcia et al. 2007).

The different climatic zones and soil types that influence soil microbial communities and their ability to acclimatise to a tillage operation may have contributed to the disparity in the impacts caused by a one-time tillage. The tillage implements in the aforementioned studies were mostly MP, and this represents the industry standards for the agricultural practices within America and Europe (López-Garrido et al. 2011; Melero et al. 2011; Wortmann et al. 2010). Tillage implements generating minimal soil inversion such as chisel, disc and Kelly chain are widely used in the northern grain-growing regions of Australia (NGR) (Dang et al. 2015). Strategic tillage as a soil management practice can influence soil microbial properties in NT agro-ecosystems. However, the tillage impacts on soil physico-chemical properties and especially the change of soil biota of the NT soils by using these implements for ST are largely unknown. In regard to addressing the insufficient knowledge about tillage effects on Australian soils, chapters 2, 3 and 4 of this thesis focused on the

effects of strategic tillage using low soil inversion implements on soil microbial properties of NT soils in NGR. The locations of the selected experimental sites, the tested factors of ST and the site management history are shown in Figure 2.

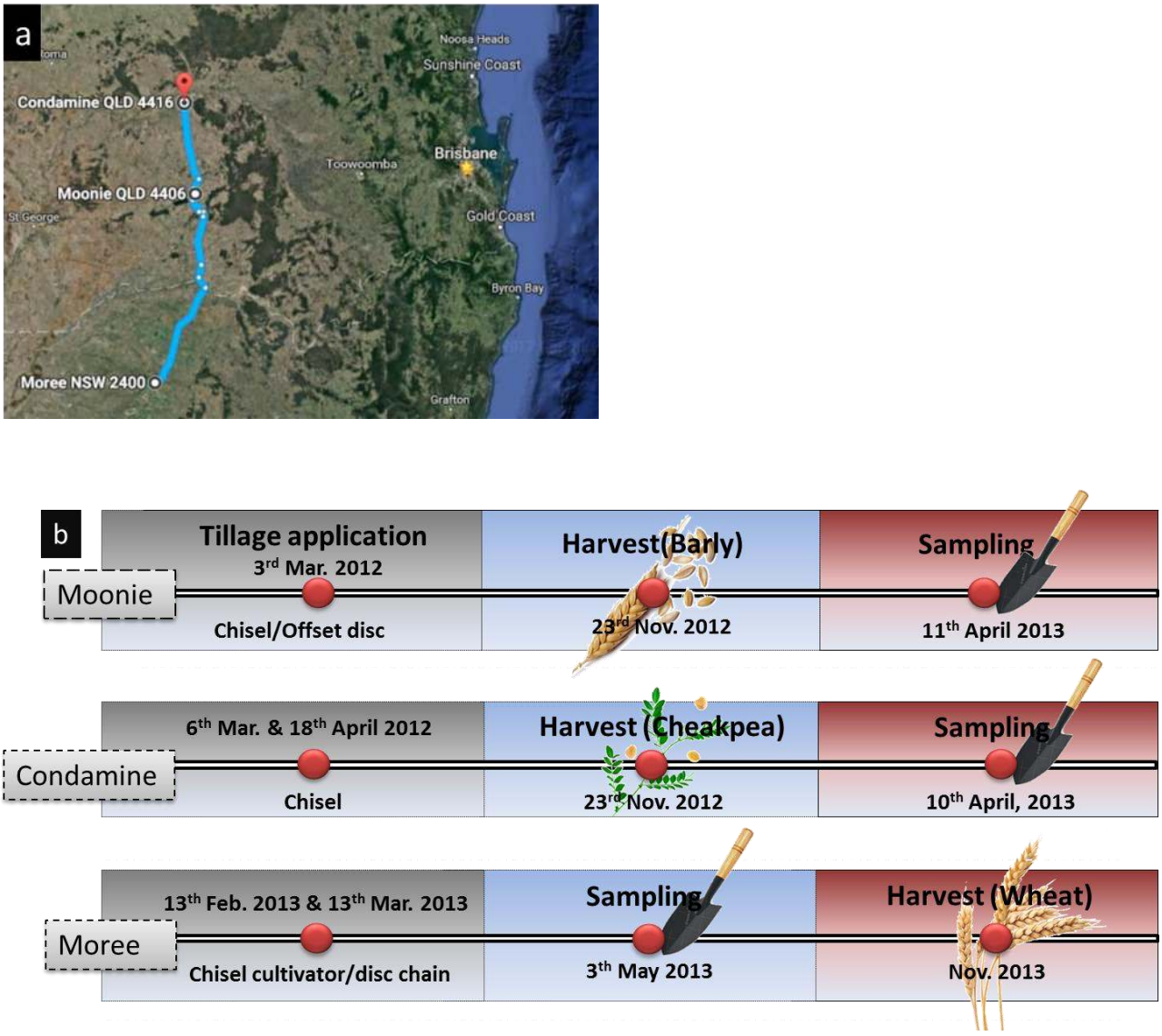

Fig. 2 Experimental sites. Site locations used in my project studying the impacts of strategic tillage (a); the design for tillage timing and implements, and the conditions for crop harvest and soil samplings in the three field trials (b).

2.5 Rhizosphere (Ectorhizosphere)

Rhizosphere is the narrow soil zone (~1 mm) surrounding plant roots (Bisseling et al. 2009). The rhizosphere has higher concentration of available nutrients than bulk soil and around 5~25% of plant synthesised C is released into rhizosphere (Bais et al. 2006). Those C compounds released into the rhizosphere come in diverse forms, including root mucilages, exudates, soluble lysates as well as sloughed-off root cells and tissues (Bais et al. 2006). Rhizosphere is the interface where plants

interplay with soil microorganisms, with 1 g rhizosphere soil containing ~10^{8-11} microbial cells. Rhizosphere bacteria can have either neutral, beneficial or antagonistic effects on plant health and performance. Those bacteria that have beneficial effects on plant growth are known as plant growth promoting rhizobacteria (PGPR), which assist in plant nutrient acquisition, and/or enhance plant tolerance to abiotic stress such as drought, salinity and coldness (Compant et al. 2005). PGPR can also elicit plant immune responses and protect plants from pathogen attacks (Berendsen et al. 2012). Studies on rhizosphere microbial properties have steadily received interest in recent decades and have shed light on roadmaps towards sustainable agriculture and phytoremediation of environmental pollutants (Berendsen et al. 2012).

2.5.1 Rhizosphere microbial properties differ from those in bulk soil

Soil properties at the microbial community level differ significantly between rhizosphere and bulk soil in terms of many microbial aspects. Firstly, rhizosphere may have distinct structure of microbial communities from that in bulk soil. By profiling bacterial and archaeal communities in rhizosphere and bulk soil, it was observed that rhizosphere often has higher relative abundance of Gamm-Proteobacteria and lower abundance of Acidobacteria and Archaea when compared with bulk soil (Fig. 3) (Edwards et al. 2015; Sessitsch et al. 2012). One plausible explanation for this phenomenon among many others is that the high rhizospheric [O_2] may have deleterious effects on many Archaeal and Acidobacterial groups in soil (Fig. 3) (Blossfeld et al. 2011). Further, in comparison with bulk soil, higher microbial biomass and enzyme activities were observed in rhizosphere (Ai et al. 2012; Kong and Six 2012; Yang et al. 2013; Zhang et al. 2012). Rhizosphere may also be associated with a higher ratio of fungi to bacteria and fungi to Actinomycetes (Ai et al. 2012), and harbours more arbuscular mycorrhizal fungi and gram-negative bacteria (Liang et al. 2016). Plant species have significant influences on rhizosphere microbial properties including soil pH, enzyme activities and microbial biomass (Liu et al. 2012; Zhang et al. 2014). Zhou et al. (2016) reported that the diversity of soil ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) differed between non-rhizosphere and rhizosphere associated with cattail (*Typha orientalis*). This study suggests that the N circulation in the rhizosphere was more active than in non-rhizosphere soils as the *amoA* gene, and AOA and AOB showed significantly higher abundance in rhizosphere soils (Zhou et al. 2016).

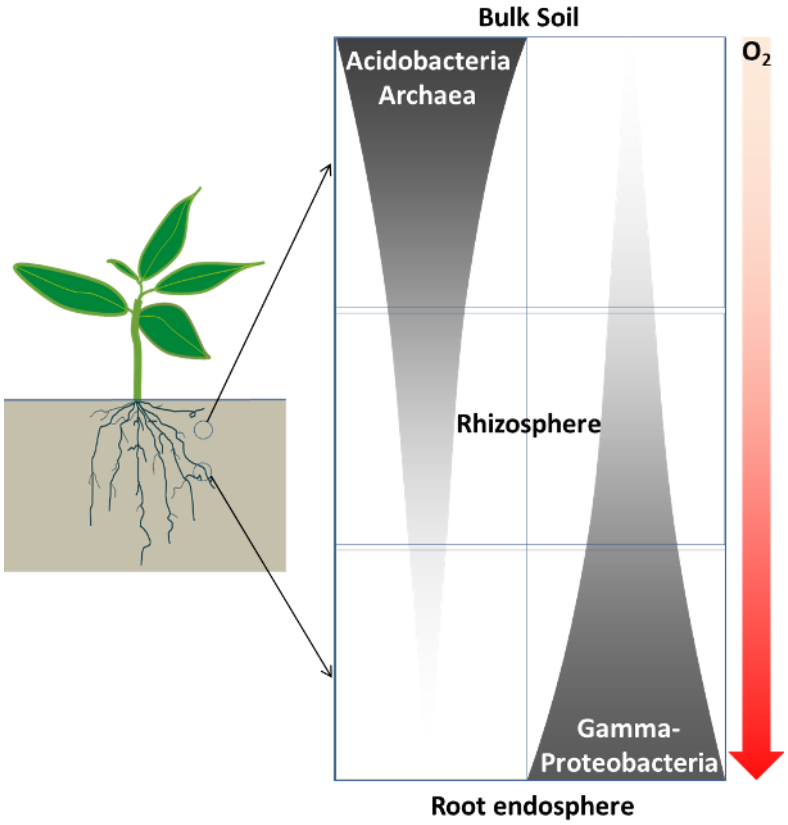

Fig. 3 A schematic graph demonstrating the enrichment of Gamma-Proteobacteria and the decrease of Archaea and Acidobacteria in plant rhizosphere and root endosphere. The red arrow represents an increasing oxygen level from bulk soil to rhizosphere and root endosphere.

2.5.2 PGPR induced plant resistance

PGPR can modulate the immune system of the plant host and induce priming in the plant that prepares them for stronger and faster defence responses. Some PGPRs, like the biocontrol agent K-165, induce resistance to *Verticillium dahiae* in *Arabidopsis* by activating JA, SA and ET signalling pathways (Tjamos et al. 2005). A number of other PGPRs like *Pseudomonas fluorescens* WCS417 and *Pseudomonas putida* WCS358 as well as plant growth promoting fungi like mycorrhizal fungi and *Trichoderma* spp. were also discovered with the ability to induce systemic resistance in plants (Van Wees et al. 2008). MYB72, a root specific transcription factor was found to be essential for the rhizobacteria-induced resistance in *Arabidopsis* (Van der Ent et al. 2008). Collectively, the plant immune system can be modulated by beneficial bacteria and fungi, by which plants are primed for accelerated defence against herbivores or pathogens.

2.5.3 Potential effects of plant signalling pathways on rhizosphere microbial communities

Long distance signalling is typically mediated by hormone molecules SA, JA and ET that translocate inside plants via the plant vascular system (Santner and Estelle 2009). Defence-related signalling pathways influence the composition of root exudates and it is intuitively to postulate that the activation of the plant immune system by challenging the plant aerial parts can re-shape the root-associated microbial communities (Badri et al. 2008). In plants, the JA signalling pathway plays a key role in plant defence against necrotrophic pathogens and herbivorous insects, which can be activated by PGPR and pathogens as well as JA and derivatives. The effects of activation of this plant defence pathway on rhizo-microbial communities have been reported inconsistently. When *Arabidopsis* was exposed to MeJA, plant defence-related microorganisms were enriched in the rhizo-microbial community, including *Bacillus* and *Lysinibacillus*-related populations (Carvalhais et al. 2013). Meanwhile, those bacterial groups that correlate to plant growth, mainly the *Pseudomonas* spp. were suppressed in the rhizosphere of *Arabidopsis*. In another study, Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis showed prominent rhizosphere effects on the composition and diversity of the microbial community (Hein et al. 2008). Furthermore, different *Arabidopsis* systemic acquired resistance (SAR) mutants showed significant differences in rhizosphere microbial community composition. But the activation of the SAR pathway by exogenous SA treatments did not change the rhizosphere diversity. Denaturing gradient gel electrophoresis (DGGE) analysis of *Arabidopsis* mutants was not sufficient to detect an influence of the JA pathway on rhizosphere bacterial community structure (Doornbos et al. 2011). Activation of induced systemic resistance (ISR) on *Arabidopsis* or tobacco by exogenous hormone treatments had no significant effects on the density and structure of the rhizosphere microbial community (Doornbos et al. 2011).

In *Arabidopsis*, it has been demonstrated that variations in SA signalling did not influence the composition of root associated bacterial communities in the rhizosphere of *Arabidopsis*. Lebeis et al. (2015) used different isogenic *Arabidopsis* mutants with altered immunity and found that root endophytic bacterial communities varied between different mutants at the family level. However, no major changes in rhizosphere bacterial communities were found in this study. This indicates that plants may require SA signalling to modulate bacterial colonisation and to drive the selection of microbial communities to sculpt root microbiomes. Yet, there are no reported studies on the interaction of plant defence signalling pathways with rhizosphere microbial communities of commercial crops, such as wheat. In the recent decade, the emergence of high throughput sequencing techniques provided a more powerful method for profiling microbial communities in environmental samples than the previous PCR-based methods of DGGE and T-RFLP. In chapters 5

and 6 of this thesis, wheat-mediated JA and SA signalling effects on wheat rhizosphere microbial communities are studied.

2.6 Endophytic bacteria interact with plants

Bacterial endophytes reside inside plant tissues without causing visible disease symptoms (Mano et al. 2008). Mounting amount of studies using genomics, metagenomics and *in vitro* analysis provide evidence that endophytic bacteria are active in plants and potentially improve plant nutrition acquisition (Moyes et al. 2016; Sessitsch et al. 2008), suppress phytopathogens (Brock et al. 2013) and increase the plant's capability in resisting biotic and abiotic stresses (Subramanian et al. 2015). Interactions between plants and endophytic bacteria have been the subject of an increasing number of studies. Widely explorations of endophytic bacteria in agricultural production have also received steadily growing interest in the recent decade. An extensive literature review on bacterial endophytes is included in **Appendix 1** of this thesis.

3 Significance of PhD study

Queensland's growers are facing a big dilemma regarding the use of ST for solving weed issues in long-term NT fields. This PhD study first examined the potential ST effects on soil microbial properties, which aimed to reveal if ST applications negate the microbial benefits of long-term NT managed soils in north-eastern Australia. Meanwhile, the ST effects on soil physico-chemical and agronomic productivity of a 19-year's NT grey Vertosol were also examined in the short-term (7-11 weeks) by collaborators. Revealing changes in soil biology caused by ST may assist Australian growers in making decisions on the use of tillage in their NT fields. The diverse array of methods that have been used in this thesis may also provide a very useful toolbox for future soil and tillage research. The values of different soil parameters gained for the different soil types at different depths are also useful for the future establishment of a database for Australian soils. Besides the factor of ST, my PhD research also determined the effects of the activation of JA and SA signalling pathways on wheat-associated microbial communities. Since the abundance and diversity of plant associated bacteria and archaea may directly or indirectly influence plant growth and health due to plant-microbe interactions, a better understanding of the factors influencing microbial diversity and composition may contribute to future sustainable agriculture. Studies on the effects of the activation of plant defence signalling on plant-associated microbial communities may also pave a way to manipulate microbial communities in wheat rhizosphere and endophytic compartments. Similarly,

crop breeding programs may focus on genetic factors to enable optimised plant-microbe interactions. Previous related studies were only performed on *Arabidopsis* and inconsistent results have been reported. The present study on wheat may complement the studies on *Arabidopsis* and shed light to better wheat production in the future.

4 References

Ai, C., Liang, G., Sun, J., Wang, X., Zhou, W., 2012. Responses of extracellular enzyme activities and microbial community in both the rhizosphere and bulk soil to long-term fertilization practices in a fluvo-aquic soil. Geoderma 173, 330-338.

Badri, D.V., Loyola-Vargas, V.M., Du, J., Stermitz, F.R., Broeckling, C.D., Iglesias-Andreu, L., Vivanco, J.M., 2008. Transcriptome analysis of *Arabidopsis* roots treated with signaling compounds: a focus on signal transduction, metabolic regulation and secretion. New Phytol 179(1), 209-223.

Bai, Y., Müller, D.B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P.C., Spaepen, S., Remus-Emsermann, M., Hüttel, B., McHardy, A.C., Vorholt, J.A., Schulze-Lefert, P., 2015. Functional overlap of the *Arabidopsis* leaf and root microbiota. Nature 528(7582), 364-369.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57, 233-266.

Berendsen, R.L., Pieterse, C.M., Bakker, P.A., 2012. The rhizosphere microbiome and plant health. Trends Plant Sci 17(8), 478-486.

Bisseling, T., Dangl, J.L., Schulze-Lefert, P., 2009. Next-generation communication. Science 324(5928), 691-691.

Blevins, R., Cook, D., Phillips, S., Phillips, R., 1971. Influence of no-tillage on soil moisture. Agron J 63(4), 593-596.

Blossfeld, S., Gansert, D., Thiele, B., Kuhn, A.J., Lösch R., 2011. The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of *Juncus* spp. Soil Biol Biochem 43(6): 1186-1197.

Brock, A. K., Berger, B., Mewis, I., and Ruppel, S., 2013. Impact of the PGPB *Enterobacter radicincitans* DSM 16656 on growth, glucosinolate profile, and immune responses of *Arabidopsis thaliana*. Microb Ecol 65, 661-670.

Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 3-22.

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

Cao, Y., Zhang, Z., Ling, N., Yuan, Y., Zheng, X., Shen, B., Shen, Q., 2011. *Bacillus subtilis* SQR 9 can control Fusarium wilt in cucumber by colonizing plant roots. Biol Fert Soils 47(5), 495-506.

Carvalhais, L.C., Dennis, P.G., Badri, D.V., Tyson, G.W., Vivanco, J.M., Schenk, P.M., 2013. Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. PLoS One 8(2), e56457.

Carvalhais, L.C., Muzzi, F., Tan, C.H., Hsien-Choo, J., Schenk, P.M., 2014. Plant growth in *Arabidopsis* is assisted by compost soil-derived microbial communities. Front Plant Sci 4, Article 235.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71(9), 4951-4959.

Dang, Y., Moody, P., Bell, M., Seymour, N., Dalal, R., Freebairn, D., Walker, S., 2015. Strategic tillage in no-till farming systems in Australia's northern grains-growing regions: II. Implications for agronomy, soil and environment. Soil Till Res 152, 115-123.

Daughtry, C., Hunt, E., McMurtrey, J., 2004. Assessing crop residue cover using shortwave infrared reflectance. Remote Sens Environ 90(1), 126-134.

Doornbos, R.F., Geraats, B.P., Kuramae, E.E., Van Loon, L., Bakker, P.A., 2011. Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. Mol Plant Microbe In 24(4), 395-407.

Edwards, J., et al., (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. P Natl Acad Sci USA 112: E911-E920.

FAO, 2014. CA Adoption Worldwide, AQUASTAT data query, ©2014 FAO of the UN, Commissioned for the exclusive use of FAO Conservation Agriculture. http://www.fao.org/ag/ca/6c.html.

Fierer, N., Bradford, M.A., Jackson, R.B., 2007. Toward an ecological classification of soil bacteria. Ecology 88(6), 1354-1364.

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. P Natl Acad Sci USA 103, 626-631.

Fierer, N., Lauber, C.L., Ramirez, K.S., Zaneveld, J., Bradford, M.A., Knight, R., 2012. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J 6(5), 1007-1017.

Garcia, J.P., Wortmann, C.S., Mamo, M., Drijber, R., Tarkalson, D., 2007. One-time tillage of no-till. Agron J 99(4), 1093-1103.

Ghimire, R., Norton, J.B., Stahl, P.D., Norton, U., 2014. Soil microbial substrate properties and microbial community responses under irrigated organic and reduced-tillage crop and forage production systems. Plos One 9, e103901.

Hein, J.W., Wolfe, G.V., Blee, K.A., 2008. Comparison of rhizosphere bacterial communities in *Arabidopsis thaliana* mutants for systemic acquired resistance. Microb Ecol 55(2), 333-343.

Ismail, I., Blevins, R., Frye, W., 1994. Long-term no-tillage effects on soil properties and continuous corn yields. Soil Sci Soc Am J 58, 193-198.

Jackson, L.E., Calderon, F.J., Steenwerth, K.L., Scow, K.M., Rolston, D.E., 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114, 305-317.

Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol 72(3), 1719-1728.

Kavoo-Mwangi, A., Kahangi, E., Ateka, E., Onguso, J., Mukhongo, R., Mwangi, E., Jefwa, J., 2013. Growth effects of microorganisms based commercial products inoculated to tissue cultured banana cultivated in three different soils in Kenya. Appl Soil Ecol 64, 152-162.

Kirkegaard, J.A., Conyers, M.K., Hunt, J.R., Kirkby, C.A., Watt, M., Rebetzke, G.J., 2014. Sense and nonsense in conservation agriculture: principles, pragmatism and productivity in Australian mixed farming systems. Agr Ecosyst Environ 187, 133-145.

Kong, A.Y.Y., Six, J., 2012. Microbial community assimilation of cover crop rhizodeposition within soil microenvironments in alternative and conventional cropping systems. Plant Soil 356(1-2), 315-330.

Lebeis, S.L., Paredes, S.H., Lundberg, D.S., Breakfield, N., Gehring, J., McDonald, M., Malfatti, S., Del Rio, T.G., Jones, C.D., Tringe, S.G., 2015. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science 349(6250), 860-864.

Liu, D., Fang, S., Tian, Y., Dun, X., 2012. Variation in rhizosphere soil microbial index of tree species on seasonal flooding land: An in situ rhizobox approach. Appl Soil Ecol 59, 1-11.

López-Garrido, R., Madejón, E., Murillo, J.M., Moreno, F., 2011. Soil quality alteration by mouldboard ploughing in a commercial farm devoted to no-tillage under Mediterranean conditions. Agr Ecosyst Environ 140(1-2), 182-190.

Lupwayi, N.Z., Rice, W.A., Clayton, G.W., 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biol Biochem 30, 1733-1741.

Mano, H., and Morisaki, H., 2008. Endophytic bacteria in the rice plant. Microbes Environ 23, 109-117.

Mathew, R.P., Feng, Y., Githinji, L., Ankumah, R., Balkcom, K.S., 2012. Impact of no-tillage and conventional tillage systems on soil microbial communities. Appl Environ Soil Sci 2012.

Matson, P.A., Parton, W.J., Power, A., Swift, M., 1997. Agricultural intensification and ecosystem properties. Science 277, 504-509.

Melero, S., Panettieri, M., Madejon, E., Macpherson, H.G., Moreno, F., Murillo, J., 2011. Implementation of chiselling and mouldboard ploughing in soil after 8 years of no-till management in SW, Spain: Effect on soil quality. Soil Till Res 112(2), 107-113.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J.H., Piceno, Y.M., DeSantis, T.Z., Andersen, G.L., Bakker, P.A., 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332(6033), 1097-1100.

Moyes, A.B., et al., 2016. Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. New Phytol 210, 657-668.

Nannipieri, P., Ascher, J., Ceccherini, M., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. Eur J Soil Sci 54(4), 655-670.

O'Donnell, A.G., Seasman, M., Macrae, A., Waite, I., Davies, J.T., 2001. Plants and fertilisers as drivers of change in microbial community structure and function in soils. Plant Soil 232, 135-145.

Palaniyandi, S.A., Yang, S.H., Zhang, L., Suh, J.-W., 2013. Effects of actinobacteria on plant disease suppression and growth promotion. Appl Microbiol Biot 97(22), 9621-9636.

Philippot, L., Raaijmakers, J.M., Lemanceau, P., Van Der Putten, W.H., 2013. Going back to the roots: the microbial ecology of the rhizosphere. Nature Rev Microbiol 11, 789-799.

Santner, A., Estelle, M., 2009. Recent advances and emerging trends in plant hormone signalling. Nature 459(7250), 1071-1078.

Sessitsch, A., et al., 2012. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol Plant Microbe Interact 25, 28-36.

Six, J., Elliott, E., Paustian, K., 2000. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. Soil Biol Biochem 32(14), 2099-2103.

Subramanian, P., Mageswari, A., Kim, K., Lee, Y., and Sa, T., 2015. Psychrotolerant endophytic *Pseudomonas* sp strains OB155 and OS261 induced chilling resistance in tomato plants (*Solanum lycopersicum* Mill.) by activation of their antioxidant capacity. Mol Plant Microbe Interact 28, 1073-1081.

Thomas, G.A., Titmarsh, G.W., Freebairn, D.M., Radford, B.J., 2007. No-tillage and conservation farming practices in grain growing areas of Queensland- a review of 40 years of development. Aust J Exp Agric 47(8), 887-898.

Thomas, F., Hehemann, J. H., Rebuffet, E., Czjzek, M., Michel, G., 2011. Environmental and gut bacteroidetes: the food connection. Front Microbiol 2:93.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J., Katinakis, P., 2005. Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. Mol Plant Microbe In 18(6), 555-561.

Unger, P., 1990. Conservation tillage systems, Advances in Soil Science. Springer, pp. 27-68.

Van der Ent, S., Verhagen, B.W., Van Doorn, R., Bakker, D., Verlaan, M.G., Pel, M.J., Joosten, R.G., Proveniers, M.C., Van Loon, L., Ton, J., 2008. MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. Plant Physiol 146(3), 1293-1304.

Van Wees, S.C., Van der Ent, S., Pieterse, C.M., 2008. Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11(4), 443-448.

Wortmann, C.S., Drijber, R.A., Franti, T.G., 2010. One-time tillage of no-till crop land five years post-tillage. Agron J 102, 1302-1307.

Yang, W., Hu, H., Ru, M., Ni, W., 2013. Changes of microbial properties in (near-) rhizosphere soils after phytoextraction by Sedum alfredii H: A rhizobox approach with an artificial Cd-contaminated soil. Appl Soil Ecol 72, 14-21.

Zhang, C., Liu, G., Xue, S., Zhang, C., 2012. Rhizosphere soil microbial properties on abandoned croplands in the Loess Plateau, China during vegetation succession. Eur J Soil Biol 50, 127-136.

Zhang, L., Song, L., Xu, G., Chen, P., Sun, J., Shao, H., 2014. Seasonal dynamics of rhizosphere soil microbial abundances and enzyme activities under different vegetation types in the coastal zone, Shandong, China. Clean Soil Air Water 42(8), 1115-1120.

Zhou, X., Zhang, J., Li, Y., Liu, B., Chu, J., Wang, M., He, Z., 2016. Distribution characteristics of ammonia oxidizing microorganisms in rhizosphere sediments of cattail. Ecol Eng 88, 99-111.

Chapter 2 One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol

Overview

No-tillage as a sustainable agriculture practice is being widely used worldwide and is associated with significant economic and environmental benefits. While NT practices benefit Australian growers, some undesirable characteristics, especially the build-up of herbicide-resistant weed populations, have become prominent in long-term NT fields. Across Australia's northern grains-growing regions, several dominant weed species have developed strong resistance to glyphosate. Some Australian growers resort to occasional tillage to control weeds instead of overusing herbicides. To address the insufficient knowledge about ST effects on Australian soils, the present chapter focuses on the effects of ST on microbial properties of a NT soil in Condamine, Australia, using different minimal soil inversion implements.

Highlights

- One-time ST was used in continuous no-till Calcisol in Australia;
- Chisel or offset disc tillage did not exert negative effects on soil biological properties;
- Chisel tillage increased soil microbial biomass carbon;
- Chisel tillage increased soil ability for degradation of D+cellubiose and mannitol
- Chisel tillage increased Alphaproteobacteria, Bacteroidetes and Firmicutes.

One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol

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Abstract

Strategic tillage (or occasional tillage) has been touted as a potential solution for the severe weed infestations of long-term no-till (NT). Nevertheless, there is little information on the influence of strategic tillage on microbial properties of Australian NT soils. In the present study, we assessed the influence of strategic tillage on the microbial properties of a seven years' NT Calcisol in Moonie, Queensland, Australia. We tested the hypothesis that the application of one-time strategic tillage with chisel or offset disc does not cause major impacts on soil biological health in a NT system. Strategic tillage was applied once and soil samples were collected 13 months after tillage from the depths 0-10 and 10-20 cm. The measured biological indicators included soil microbial biomass carbon, catabolic activity (MicroResp^{TM} assay) and total microbial activity (fluorescein diacetate method). The structure of bacterial communities was profiled by quantitative polymerase chain reaction (qPCR) and terminal reaction fragment length polymorphism (T-RFLP). Principal components analyses based on qPCR and T-RFLP data did not show tillage effects on soil bacterial communities. However, relative to the NT, chisel tillage led to significant increases in microbial biomass carbon (+34.4%), abundances of Alphaproteobacteria (+74.6%), Bacteroidetes (+113.7%) and Firmicutes (+36.5%), and the utilisation of D+ cellubiose (+178.4%) as well as mannitol (+167.2%) at 0-10 cm depth. In contrast, the influence of offset disc tillage was restricted to an increased abundance of Alphaproteobacteria (+64.6%) at 0-10 cm depth. Our study suggests that, overall, one-time strategic tillage using either chisel or offset disc had a minor positive influence on soil biological attributes of the NT Calcisol 13 months after tillage.

Keywords: bacterial genetic fingerprinting; soil microbial activity; conservation agriculture; Dermosol; Northern Grains Region of Australia

1 Introduction

No tillage, also known as zero tillage or direct drilling, has been widely adopted worldwide, and especially in Australia. No-till (NT) was applied in 17,695,000 ha Australia-wide in 2014, accounting for 11.4% of the world's NT cropping lands (FAO, 2014). Over the whole state of Queensland (QLD), NT represents approximately 50% of its total cropping land, but the rate could be up to 85% in some regions of QLD (Thomas et al., 2007).

As a method of conservation farming, NT favors sustainable agriculture and is associated with many environmental, social and economic benefits (Derpsch et al., 2010). Previous studies have reported that conservation agriculture is typically associated with higher microbial biomass in the top soil layer compared with conventional tillage (Govaerts et al., 2007; Madejón et al., 2009; Page et al., 2013). Soil microbial activity, which can be measured by soil enzymatic activity, also tends to be enhanced by NT (Mathew et al., 2012; Sharma et al., 2013). Roldán et al. (2005) reported that dehydrogenase (+46.2%, 0-10 cm), protease (+178.0%, 0-20 cm), β-glucosidase (+122.2%, 0-5 cm), urease (+63.1%, 0-10 cm) and phosphatase (+59.0%, 0-20 cm) exhibited higher activity in a three years' NT Vertisol over soils tilled by mouldboard plough (MP). Additionally, NT in combination with residue retention was found to promote beneficial bacteria such as *Pseudomonas* spp., *Rhodospirillales* and *Burkholderiales* (Ceja-Navarro et al., 2010). Some other environmentally important bacterial groups such as Actinobacteria (+81.8%, 0-15 cm) and Rhizobiales (e.g. *Methylosinus* spp.) (+434.5%, 0-15 cm) were also present at higher abundances in NT (Ceja-Navarro et al., 2010; Mathew et al., 2012). These bacteria are involved in the mineralisation of soil organic residues, which is essential for carbon and nitrogen cycling in agro-ecosystems (Sharma, 2014). Stubble retention and minimum soil disturbance appear to have contributed to the agricultural improvements achieved by NT (Hobbs et al., 2008).

Despite all the benefits gained with NT, concerns have arisen among farmers about the outbreak of herbicide-resistant weed populations and the increased prevalence of stubble-borne diseases in Australian NT (Llewellyn et al., 2002). Weed control in Australian NT is presently highly reliant on chemical herbicides, and the continuous use of these chemicals has led to multiple herbicide-resistant weeds (D'Emden et al., 2008). A survey conducted in 2008 by the Australian Grains Research & Development Corporation depicted that a substantial increase in the price of glyphosate led to 21% of Australian growers increasing the usage of occasional tillage for weed control (Llewellyn and D'Emden, 2010). However, little is known about the impact of occasional tillage on microbial properties of the NT soils in Australia.

Many studies outside Australia have shown unfavourable effects of occasional tillage on soil biological properties. Studies conducted by López-Garrido et al. (2011) and Melero Sánchez et al. (2011) reported that one-time tillage with MP reduced various biological indicators of soil health in the top 5 cm layer of soil, including soil microbial biomass carbon (by 19%) and nitrogen (by 44%), and microbial enzymatic activities such as dehydrogenase (by 40%), beta-glucosidase (by 50%) and diphenol oxidase (by 14%). One-time tillage applied to a NT clay loam soil also reduced root colonisation of arbuscular mycorrhizal fungi by 58 to 87% (Garcia et al., 2007). Additionally, distinct responses of NT soils to one-time tillage were documented five years after tillage with a MP between two experimental sites: one site (Sharpsburg silty clay loam soil) showed a decline in microbial biomass and a shift of microbial community structure while at the other site (Yutan silty clay loam soil) these parameters showed no difference from the NT (Wortmann et al., 2010). Other studies have reported no negative effect of strategic tillage in NT (Crawford et al., 2015; Wortmann et al., 2008). Such disparities in the impacts caused by an occasional tillage can be attributed to different sampling times, climates and soil types which influence soil microbial communities and their ability to adapt to tillage.

The common theme in the aforementioned studies is the use of a big inversion implement of MP. These findings listed above are less applicable to the agriculture in northern Australia, where most tillage involves chisel and disc implements instead of MP (Dang et al., 2015). For this reason, it is imperative to determine the influence of low inversion tillage on soil microbial attributes in Australian agro-ecosystems. Crawford et al. (2015) reported that the in-crop weed populations were reduced at 3 months (chisel, - 89.2%; offset disc, - 86.5%) and 13 months (chisel, - 66.7%; offset disc, - 82.7%) after one-time strategic tillage using these two implements in an experimental site located in Moonie, Queensland, Australia (27.79°S, 150.20°E). However, important biological attributes associated with soil health were not analysed in this study.

The objective of the present study was to examine the influence of chisel and offset disc tillage on soil microbial properties of this long-term NT Calcisol at Moonie. We tested the hypothesis: ‘One-time strategic tillage using less inversion implements does not cause major impacts on biological attributes of NT soils’. The lasting tillage effects on soil biological parameters after one harvest of wheat in an experimental field were determined. Revealing changes in biology caused by tillage after cropping will provide valuable information to farmers, and will assist them in making decisions on the use of strategic tillage.

2 Materials and Methods

2.1 Site descriptions, tillage application and sample collection

The experimental site was located 16 km south-west of Moonie QLD (27.79°S, 150.20°E), in the Northern Grains Region of Australia. Soil at this site had not been tilled for seven years and is classified as a Calcisol (World Reference Base (WRB), IUSS 2007), or Sodic Hypocalcic Grey Dermosol (Australian Soil Classification (ASC)). Wheat (*Triticum aestivum* L.) was previously cultivated at this site during the seven years of NT. A summary of chemical properties and granulometric fractions of Moonie soils are described in Table 1. To avoid repetition, a detailed site description can be found in Crawford et al. (2015).

Table 1 Granulometric fractions and chemical properties of Moonie soils at 0-10 and 10-20 cm depths.

Component	EC	pH	TOC	P	NH_4-N air dry	NO_3-N air dry	Ca	Mg	Na	K	ESP	CEC	Clay	Silt	Sand
Units	dS m^{-1}	-	g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	meq 100 g^{-1}	meq 100 g^{-1}	meq 100 g^{-1}	meq 100 g^{-1}	%	meq 100 g^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
0-10 cm	0.18	8.7	8.47	17.2	3	23	8.28	10.2	3.14	0.389	17.7	18	312	101	587
10-20 cm	0.20	8.9	5.45	3.25	4	14	15.1	7.71	1.71	0.754	8.6	20	378	102	520

EC: electrical conductivity; TOC, total organic carbon; ESP: exchangeable sodium percentage; CEC: cation-exchange capacity.

The field experiment was arranged as a randomised block design (100 x 12 m) with four replicates per treatment. Tillage treatments included: (i) NT; (ii) one-time chisel tillage; and (iii) one-time offset disc tillage. Tillage with both chisel (37.5 cm between tines) and offset disc were performed to a depth of 10 cm on the 3rd of March 2012. This site was used for the cultivation of barley (*Hordeum vulgare* L.) after the strategic tillage, and thirteen months after tillage was considered a suitable short-term assessment. Seven sub soil samples of approx. one kg per plot were collected between previous barley seeding lines using a hand shovel on the 11th of April 2013 (139 days after the harvest of barley when the plot was in fallow) from the depth of 0-10 cm and 10-20 cm. These seven samples were collected along an imaginary Z shape with a covering area of 90 m x 10 m and composited according to soil depth. Soil samples were sieved (< 4 mm) and assessed for gravimetric water content immediately before being stored at 4°C for further tests. Tillage, site location, weed infestations and weather in the experimental site were noted as shown in Fig. 1

(A~G). As previously reported, in-crop weeds were significantly suppressed at both 3 and 13 months after one-time strategic tillage with chisel or offset disc (Crawford et al., 2015).

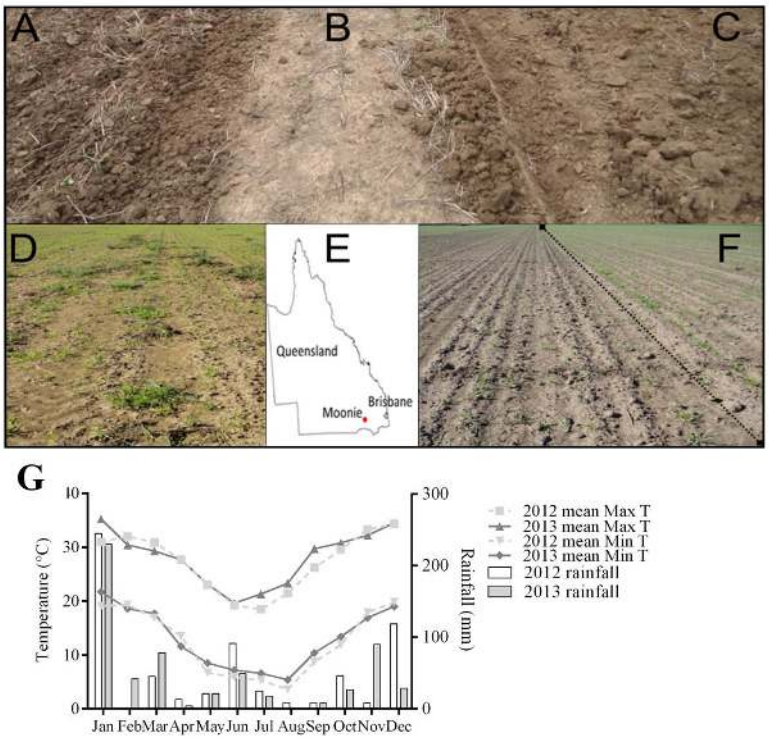

Fig. 1 Experimental site. A, chisel tilled soils; B, no-till (NT) soils; C, offset disc tilled soils (soil pictures of A, B, C were taken on the date of tillage); D, weed infestations in NT soils 3 months after tillage; E, location of the experimental site; F, weed infestation in chisel (bottom left) and offset disc (upper right) tilled soils three months after tillage; G, rainfall and temperature at the experimental site.

2.2 Microbial biomass carbon

Microbial biomass carbon was determined using the method of chloroform fumigation-extraction (Joergensen and Brookes, 1990). Briefly, 10 g of oven-dry equivalent fresh soil was placed on glass plates and fumigated with chloroform in a sealed glass desiccator which was then placed in a laminar flow cabinet for 72 h while a complementary set of soils was prepared without fumigation. Soluble carbon was extracted from fumigated and non-fumigated soils in 0.5 M K_2SO_4 at 250 rpm, 25ºC for 1 h. After centrifugation at 2,500 g for 10 min, total dissolved organic carbon of the supernatant was determined on a total organic carbon analyser (TOC-VCSH, Shimadzu Corp., Japan). Microbial biomass carbon was calculated according to the formula $C=E_C/k_{EC}$, where E_C= (organic C of fumigated samples) - (organic C of non-fumigated sampels), and $k_{EC} = 0.45$.

2.3 Composition and genetic fingerprints of soil bacterial communities

2.3.1 DNA extraction

Genomic DNA was extracted from sonicated soil samples using a PowerSoil® DNA Isolation Kit (MOBIO Laboratories, CA). Briefly, 5 g of fresh soil was combined with 5 mL of phosphate-buffered saline (pH 7.2). Samples were vortexed and then sonicated at a frequency of 20 kHz with 40% of maximum output for 5 min. After sonication, 2 mL of the resulted slurry was centrifuged at 13,000 g for 10 min and 250 mg of the precipitation was transferred to the glass bead tube supplied with the kit. The remaining steps were performed according to the manufacturer's instructions. DNA was quantified on a Qubit™ fluorometer using Quant-iT dsDNA HS Assay Kits (Invitrogen).

2.3.2 Quantification of bacterial groups

The relative abundances of Bacteroidetes, Alphaproteobacteria, Gammaproteobacteria, Firmicutes and Actinobacteria were determined in triplicate for each soil DNA sample by quantitative polymerase chain reaction (qPCR) using group-specific primers that target the 16S rRNA gene as previously described (Bacchetti De Gregoris et al., 2011). Briefly, group-specific relative abundances were determined by comparing the quantity of amplicons obtained with primers which are specific to certain bacterial taxa to the ones generated from total bacteria using universal primers. PCR on a Light Cycler® 96 (Roche) contained 1×Faststart SYBR green mix (Roche Diagnostics Ltd), 3 µL of each primer (0.3 µM), and 5 ng of DNA template in a 13 µL volume. PCR conditions comprised initial denaturing at 95°C for 10 min; 30 cycles of 95°C for 15 s, 61°C for 15 s, and 72°C for 20 s; final elongation 72°C for 5 s. Melting curves were obtained at 95°C for 10 s, 65°C for 60 s and 97°C for 1 s. The obtained data were subsequently processed by the program provided by Light Cycler® 96 (Roche). Prior to the qPCR, inhibition tests were performed to ensure no inhibition of target amplification. Briefly, three random DNA samples from the same soil depth were combined, and sequentially diluted to concentration 5 ng μL^{-1} and 2.5 ng μL^{-1}, 1 µL of which was used in qPCR (Fig. S2).

2.3.3 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Bacterial 16S rRNA genes were PCR amplified using the primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3'). The forward primer was labelled with the fluorophore 6-FAM at the 5' end. PCR was performed in a 25 µL reaction mixture containing: 14.75 µL of ultra-pure water, 5 µL of 5×Phire buffer (Thermo Scientific), 1.25 µL of dNTPs (10 µM), 1.25 µL of a 10 µM fluorescently-labelled 27F, 1.25 µL of a

1492R (10 μM), 0.5 μL of Phire® hot-start II (Thermo Scientific), and 1 μL of DNA template (10 ng μL^{-1}). PCR conditions were 30 s at 98°C for initial denaturation, 29 cycles of 15 s at 98°C, 30 s at 56°C for annealing and 45 s at 72°C; followed by 7 min at 72°C for final extension. Amplifications were performed in duplicate on each sample and the amplified PCR products were confirmed by 1% agarose gel electrophoresis, and then combined. An aliquot of 20 μL was subsequently digested using incubation with 1 U *Msp*I at 37°C for 2 h, and the digested PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). All digested PCR samples were adjusted to 50 ng μL^{-1} before being sent to AGRF (Australian Genome Research Facility Ltd., Melbourne) for fragment length analysis using capillary electrophoresis (AB3730 DNA analyser).

2.4 Microbial activity assays

2.4.1 Carbon substrate utilisation

Carbon substrate utilisation was measured with the MicroResp™ assay (Campbell et al., 2003). A total of fourteen carbon sources were used as substrates, including water as a control as shown in Table 2. In triplicate, 0.5 g of soil was distributed at the bottom of each deep well, and the water content of soil was adjusted to 300 g kg^{-1} by adding milli-Q water into each deep well. Soil samples were incubated at 25°C for 3 days in a sealed plastic box containing a dish of self-indicating soda lime. Each carbon substrate was dissolved in water to a concentration that allowed addition at the rate of either 7.5 mg or 30 mg per gram of soil at 30% of the soil's water-holding capacity (Campbell et al., 2003) (Table 2). An indicating plate that was fixed on deep well plate contained 1% agar, 150 mM KCl, 2.5 mM $NaHCO_3$ and 12.5 μg mL^{-1} cresol red. Absorbance of the indicating plate at 570 nm was measured on a plate reader (BMG Lab, Ortenberg, Germany) before and after 6 h incubation at 25°C. CO_2 production rate (μg CO_2-C g^{-1} h^{-1}) was calculated using the difference between measurements at these two time points.

Table 2 Carbon substrates used in soil metabolic activity assessment.

Group	Carbon Sources	Concentration (mg g^{-1} soil H_2O)
Carboxylic acids	Citric acid	30
	L-malic acid	30
	Methyl pyruvate	30
	Oxalic acid	7.5
	D+ galacturonic acid	7.5
Carbohydrates	Mannitol	7.5

	β-d-fructose	30
	D-(+)- trehalose	30
	D-glucose	30
	D+ cellubiose	7.5
	D-xylose	7.5
Amino acids	L-alanine	7.5
	γ-aminobutyric acid	7.5
Polymer	Tween 80	7.5

2.4.2 Total microbial activity

Total microbial activity was determined in triplicate using the fluorescein diacetate hydrolysis (3, 6-diacetylfluorescein, FDA) assay (Green et al., 2006). To a mixture of 15 mL potassium phosphate buffer (60 mM, pH 7.6) and 2 g of soil in a sterile Falcon tube, 200 µL of a 2 mg mL^{-1} FDA solution was added as substrate and shaken at 150 rpm at 30 °C for 1 h. An aliquot of 950 µL from each reaction was mixed thoroughly with an equal volume of 2: 1 (v: v) chloroform: methanol to stop the reaction, and subsequently centrifuged at 12,000 g for 3 min. The absorbance of 250 µL of supernatant was read at 450 nm in a plate reader (BMG Lab, Ortenberg, Germany).

2.5 Statistical analysis

The effects of tillage and depth on microbial biomass carbon, FDA hydrolysis and the utilisation of individual carbon substrates were investigated using ANOVA with post hoc comparison of means using the least significant difference (LSD). The effects of tillage and depth on utilisation of carbon substrates and composition/genetic fingerprints of bacterial communities were investigated using PERMANOVA. Principal component analysis (PCA) and/or heatmaps were generated to visualise differences in the genetic fingerprints of bacterial communities or the utilisation of carbon substrates between samples.

3 Results

3.1 Microbial biomass carbon

As shown in Fig. 2, the main effect of tillage was not statistically significant; however, there was a significant interaction between tillage and sampling depth (P = 0.016, ANOVA). This interaction was attributed to a slight increase in microbial biomass carbon (P = 0.042, +34.40%) by chisel tillage compared with the NT at 0-10 cm depth. At 10-20 cm depth, however, chisel tillage

did not influence microbial biomass carbon relative to the NT. Offset disc tillage did not influence microbial biomass carbon at either depth compared with the NT. On average, microbial biomass carbon was greater in soils sampled from 0-10 cm depth than in those sampled from 10-20 cm depth (Fig. 2; $P < 0.001$, ANOVA).

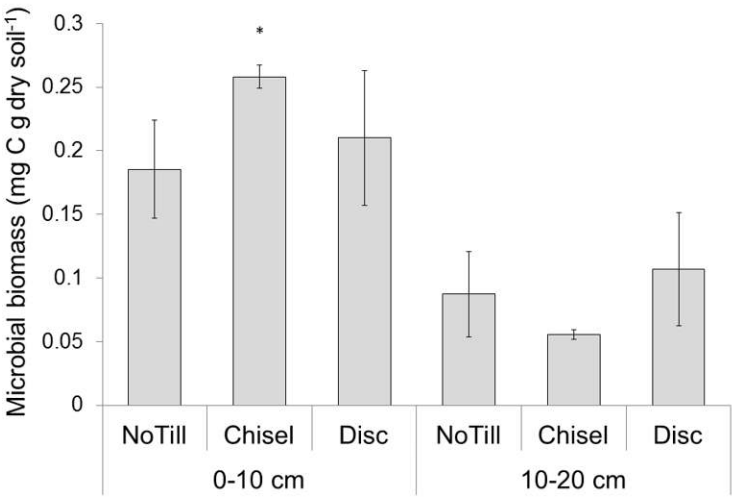

Fig. 2 Microbial biomass carbon. Shown are mean values (n=4) with SDs as error bars. The asterisk represents a statistically significant difference in comparison to the NT.

3.2 Composition and genetic fingerprints of bacterial communities

Changes in bacterial communities in response to chisel tillage at 10-20 cm depth or offset disc tillage at either depth were not detected by using the methods of qPCR and T-RFLP to characterise such communities (Figs. 3 A, B). A marginally significant interaction between tillage and sampling depth was detected using the qPCR data (Fig. 3A, $P = 0.078$, PERMANOVA). This effect was related to an enrichment of Firmicutes ($P = 0.034$, +36.49%) and Bacteroidetes ($P = 0.018$, +113.76%) by chisel tillage and Alphaproteobacteria by both chisel ($P = 0.021$, +113.76%) and offset disc ($P = 0.039$, +64.58%) tillage at 0-10 cm depth relative to the NT (Fig. S1A), but was not supported by the T-RFLP analysis (Fig. 3B). PCA revealed that, along the first axis which explains 62.4% of the variation between treatments, the bacterial taxa Bacteroidetes, Alphaproteobacteria and Gammaproteobacteria contributed to the separation of the soil profile 0-10 cm; while Actinobacteria contributed to the separation of the soil profile 10-20 cm (Fig. 3A). Both qPCR and T-RFLP analysis of different bacterial groups showed that the composition of bacterial communities differed between depths (Fig. 3; $P < 0.001$, PERMANOVA). Using 1 µL of DNA dilutions of 5 ng μL^{-1} and 2.5 ng μL^{-1} in qPCR resulted in a linear relationship between Ct values and $\log_2$ (DNA dilution folds) for all six primers tested (Fig. S2), indicating no inhibition of target amplification.

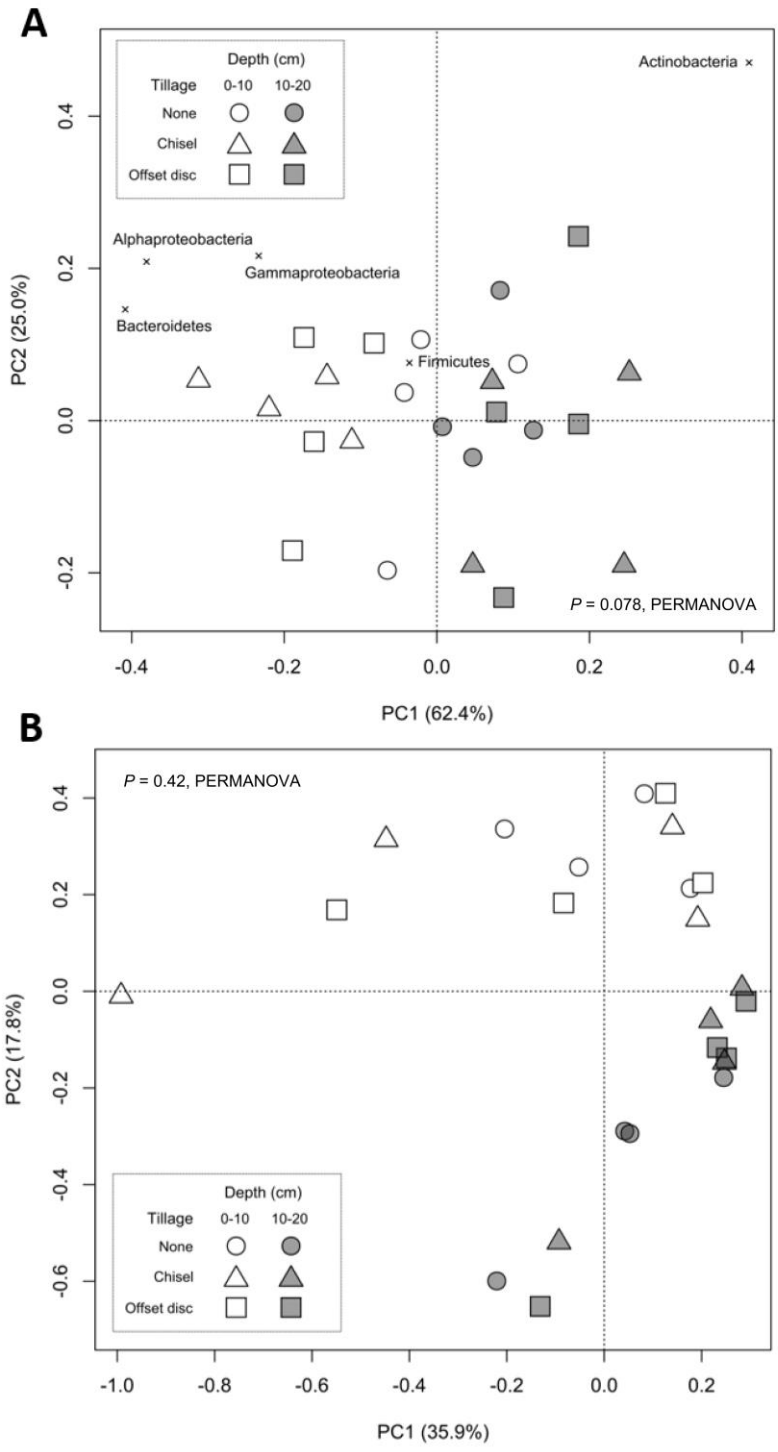

Fig. 3 Principal component analysis (PCA) ordination summarising variation in the composition of bacterial communities between samples as indicated by the qPCR analysis of five bacterial groups (A) and T-RFLP analysis of full-length 16S rRNA gene amplicons (B).

3.3 Microbial activity

The MicroResp^{TM} physiological profiles analysed by PCA are shown in Fig. 4 A. The PC1

and PC2 explained 48.4% and 18.5% of the total variance, respectively. Overall utilisation of carbon substrates was not influenced by tillage (Fig. 4 A; P = 0.18, PERMANOVA), although typically greater measurements were recorded in the 0-10 cm depth than in the 10-20 cm depth (Fig. 4 A, B; P < 0.001, PERMANOVA). When each carbon substrate was analysed independently, all but two (citric acid and oxalic acid) were found to be utilised more rapidly in soil from 0-10 cm depth than soil from 10-20 cm depth. In addition, two substrates, D+ cellubiose (P = 0.014, +178.4%) and mannitol (P = 0.029, +167.2%), were shown to be influenced by tillage, although this effect was only apparent between the chisel tilled soils and NT soils at 0-10 cm depth. Utilisation of xylose was marginally higher in chisel tilled soils (P = 0.058) compared with NT soils. When the utilisation of all fourteen carbon substrates was considered together, the rate in chisel tilled soils was significantly higher than in NT soils (Fig. 4 B, P = 0.033, +72.9%).

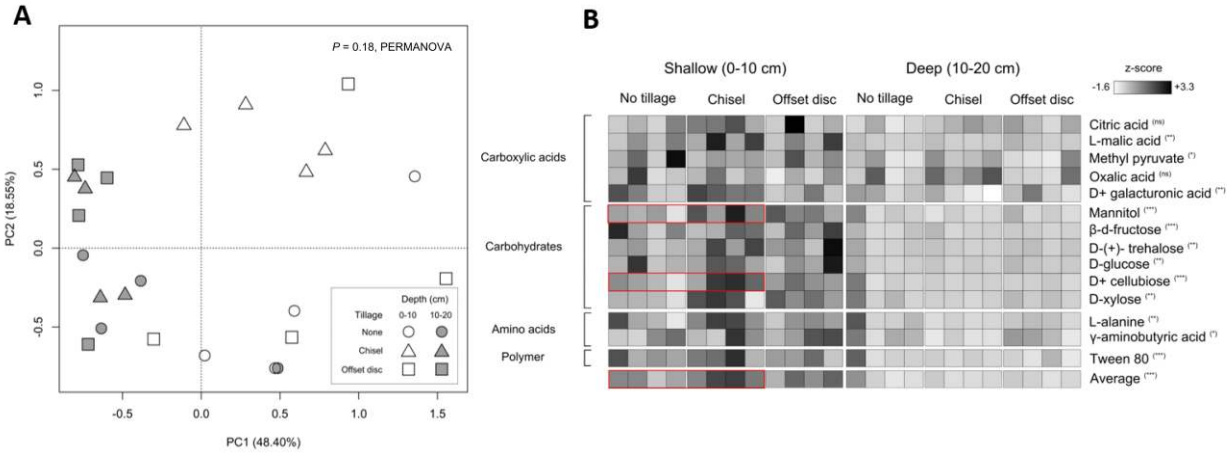

Fig. 4 Principal component analysis (PCA) ordination (A) and heatmap (B) summarising variation in the substrate utilisation profiles between samples based on the z-score transformed C-utilization (CO_2 evolution) data. The significance of this effect is reflected by the asterisks following the name of each carbon substrate (P > 0.05 $^{(ns)}$, P < 0.05 $^{(*)}$, P < 0.01 $^{(**)}$, P < 0.001 $^{(***)}$). The red boxes indicate that the utilisation rates of D+ cellubiose, mannitol and average utilisation of carbon substrates were significantly greater in the chisel-tilled soils (9.02 µg CO_2-C g^{-1} h^{-1}, 1.75 µg CO_2-C g^{-1} h^{-1} and 8.42 µg CO_2-C g^{-1} h^{-1}, respectively) when compared with the NT (3.24 µg CO_2-C g^{-1} h^{-1}, 0.65 µg CO_2-C g^{-1} h^{-1} and 4.87 µg CO_2-C g^{-1} h^{-1}, respectively) at 0-10 cm depth.

No effects of tillage were detected for total microbial activity, as indicated by the rates of FDA hydrolysis. However, greater rates were observed at 0-10 cm depth than 10-20 cm depth (Fig. 5, P < 0.001, ANOVA).

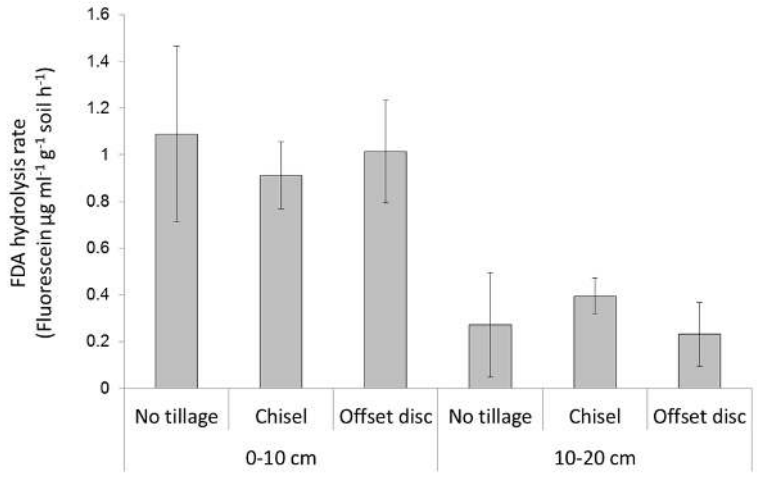

Fig. 5 Total microbial activity as indicated by FDA hydrolysis rates. Shown are mean values (n=4) with SDs as error bars.

4 Discussion

In general our results support our hypothesis, given that only slight increases of the tested soil microbial parameters in the NT were found after one-time strategic tillage. Overall, the major biological attributes tested in NT soils were not impacted by strategic tillage after one harvest of wheat crop in the experimental field.

4.1 Effects of strategic tillage on soil microbial biomass carbon

Microbial biomass carbon is a major component of the active soil organic matter pool, which regulates nutrient and energy cycling in soils (Wardle, 1992). Offset disc and chisel implements differ in their ways of disturbing soil physical structures. Offset disc is an implement that causes substantial breakdown of soil aggregates and mainly influences superficial soil profiles (<10 cm) (Raper, 2002). In contrast, chisel-type implements have narrow points, being designed not to invert the soil profiles and can be adjusted to till deep or shallow soil layers. Chisel-type implements change the soil structure by initial loosening or aerating the soil while leaving most of organic residues on the soil surface. In the present study, the microbial biomass carbon: total organic carbon (MBC: TOC) ratio which indicates the soil efficiency in converting organic carbon to microbial biomass carbon was slightly higher in the chisel tilled soils (8.30%) than NT soils (6.34%) (Crawford et al., 2015). This indicates that an environment more conducive to enhanced microbial biomass carbon may have been produced by chisel tillage on this particular soil type at that given time.

The type of implement utilised plays a pivotal role in determining the effects of occasional tillage on the soil microbial biomass. Generally, tilling with an offset disc or chisel is reported to cause less soil disturbance than that of the commonly used MP in European/US traditional tillage (Conant et al., 2007; Dang et al., 2015). According to previous reports, tillage with MP typically decreases soil microbial biomass (-13.70% ~ -40.63%) in shallow soils (López-Garrido et al., 2011; Melero et al., 2011; Wortmann et al., 2010; Wortmann et al., 2008). Consistent with our study, Melero et al. (2011) also reported that soil microbial biomass carbon was increased by 46.59% at 0-5 cm depth in a clay loam soil eight months after chisel tillage, sampling the soil after harvesting a wheat crop.

4.2 Effects of strategic tillage on the structure of bacterial communities

Determining the composition of higher taxa in bacterial communities by qPCR using taxa-specific 16S rRNA primers is an effective method for discriminating between different land use and soil managements (Bacchetti De Gregoris et al., 2011). In this study, the relative abundances of five different taxa within the total bacterial communities were obtained. In general, strategic tillage using chisel and offset disc did not cause major influence on the composition of bacterial communities. The changes caused by chisel tillage included the increased abundances of Firmicutes, Bacteroidetes and Alphaproteobacteria at the 0-10 cm depth, which is in agreement with the corresponding increase of microbial biomass carbon. The phylum Bacteroidetes is diverse and includes many populations that participate in the carbon cycle by decomposing organic compounds in the biosphere (Thomas et al., 2011). Bacteroidetes are well-known degraders of polymeric organic matter, especially polysaccharides and proteins (Mayrberger, 2011). Alphaproteobacteria is another diverse order which comprises some environmentally or agriculturally important bacteria such as rhizobiales which can be symbiotic and assist plants in acquiring nitrogen through nitrogen fixation (Ceja-Navarro et al., 2010).

Since the experimental site has a long history of NT and wheat cropping, high resistance and/or resilience to soil disturbance may have been established at the time of tillage, which could have led to the minimal impacts as observed in this study (Kibblewhite et al., 2008). This is not uncommon as previously reported for a 25-year wheat field where no differences in community structure based on ester-linked and phospholipid-linked fatty acid methyl esters (FAMEs) were observed between reduced tillage and NT (Drijber et al., 2000). However, it is important to consider that tillage may have impacted the tested biological indicators within the year between tillage and soil sampling. Furthermore, chisel and offset disc tillage may have caused low pressure on bacterial communities due to the low level of soil inversion produced, and thus soil bacterial communities

may have rapidly recovered to the NT conditions one-year after tillage. Similar to our findings, Wortmann et al. (2008, 2010) reported that one-time tillage using mini-MP in a silty clay loam soil in eastern Nebraska (USA) allowed the structure of soil microbial community recovering to pre-disturbance conditions in a one-year time frame while the soil tilled by MP took three years to recover its microbial communities.

4.3 Effects of strategic tillage on soil microbial activity

4.3.1 Carbon substrate utilisation

The tillage effect as indicated by the utilisation of D+ cellubiose and mannitol was restricted to chisel tillage, which is consistent with the corresponding increase of microbial biomass carbon and the enrichment of Firmicutes, Bacteroidetes and Alphaproteobacteria seen at 0-10 cm depth. Cellubiose is a common glucose disaccharide derived from the partial hydrolysis of cellulose (Schellenberger et al., 2011), and some members of Bacteroidetes are known for decomposing cellulose and cellubiose through extracellular enzymes (Mayrberger, 2011). Therefore, the increased abundance of Bacteroidetes possibly contributed to the increased cellubiose utilisation in the chisel-tilled soils. Chisel loosened/broke down the shallow soils, which may have resulted in more oxidative biochemical environments than NT soils and slightly increased the utilisation of carbon substrates (Melero et al., 2011). These results indicate that one-time tillage using either chisel or offset disc had a minor influence on catabolic profile of the soil microbial communities.

4.3.2 Total microbial activity

FDA hydrolysis is a simple, sensitive and rapid method in reflecting the status of a variety of soil enzymes, including esterases, lipases and certain proteases (Caldwell, 2005). This method measures the activity of enzymes located in different compartments in the soil. Esoenzymes (those are bound to the outer cell membrane), as well as peri- and intracellular enzymes are the ones that contribute to the microbial activity given that they are associated to active living microbial cells (Nannipieri et al. 2002). Extracellular enzymes (or exoenzymes) which are protected by humic compounds or stablilised by surface reactive particles are also detected by this method and can remain in the soil over longer periods of time (Nannipieri et al., 2012, 2002). Therefore, it is very important to consider the FDA data as potential microbial activity rather than the actual microbial activity.

Overall, tillage effects on the total microbial activity of the NT Calcisol were not significant irrespective of the implement types. As a broad-scale method, the FDA hydrolysis assay may not be sensitive enough to evaluate the effect of ST on specific enzymes such as cellulose and lactase.

However, the results of this assay suggested that major soil microbial functions relating to decomposition were maintained in treatments with chisel and offset disc.

4.4 Depth effects on soil microbial properties

Stratification of soil physical, chemical and biological properties could cause adverse effects on crop performances due to nutrient immobilisation at soil surface, especially under dry conditions (Mallarino et al., 1999). There have been studies conducted to reduce soil stratification in continuous NT by one-time tillage, where MP ploughing effectively redistributed nutrients while disc/chisel tillage did not (Garcia et al., 2007; Quincke et al., 2007, Wortmann et al., 2010). One-time tillage with chisel and offset disc did not seem to homogenise the soil microbial properties of the NT in the present study.

5 Conclusion

No major effect of one-time strategic tillage by either chisel or offset disc in the Calcisol under 7 years NT was observed using the biological indicators measured in our study. In conjunction with our previous findings that soil physical and chemical properties of this NT Calcisol were not influenced by one-time strategic tillage using these two implements, we provide further evidence to suggest that one-time strategic tillage with these low soil inversion implements may be suitable to tackle problems associated with continuous NT (e.g. weed infestation). Future research is needed to monitor the effects of one-time strategic tillage on other soil microbial groups, such as fungi and micro-invertebrates, as well as on different soil types and climates within Australia.

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Conflict of interest

The authors declare no conflict of interest.

References

Bacchetti De Gregoris, T., Aldred, N., Clare, A.S., Burgess, J.G., 2011. Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. J. Microbiol. Meth. 86, 351-356.

"CA Adoption Worldwide." *FAO*, 2014. Web, retrieved 10 Oct 2014. http://www.fao.org/ag/ca/6c.html.

Caldwell, B.A., 2005. Enzyme activities as a component of soil biodiversity: a review. Pedobiologia 49, 637-644.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl. Environ. Microb. 69, 3593-3599.

Ceja-Navarro, J.A., Rivera-Orduna, F.N., Patino-Zúniga, L., Vila-Sanjurjo, A., Crossa, J., Govaerts, B., Dendooven, L., 2010. Phylogenetic and multivariate analyses to determine the effects of different tillage and residue management practices on soil bacterial communities. Appl. Environ. Microb. 76, 3685-3691.

Conant, R.T., Easter, M., Paustian, K., Swan, A., Williams, S., 2007. Impacts of periodic tillage on soil C stocks: A synthesis. Soil Till. Res. 95, 1-10.

Crawford, M., Rincon-Florez, V., Balzer, A., Dang, Y., Carvalhais, L., Liu, H., Schenk, P., 2015. Changes in the soil quality attributes of continuous no-till farming systems following a strategic tillage. Soil Res. 53, 263–273.

Dang, Y., Moody, P., Bell, M., Seymour, N., Dalal, R., Freebairn, D., Walker, S., 2015. Strategic tillage in no-till farming systems in Australia's northern grains-growing regions: II. Implications for agronomy, soil and environment. Soil Till. Res. http://dx.doi.org/10.1016/j.still.2014.12.013

D'Emden, F.H., Llewellyn, R.S., Burton, M.P., 2008. Factors influencing adoption of conservation tillage in Australian cropping regions. Aust. J. Agr. Resour. Ec. 52, 169-182.

Derpsch, R., Friedrich, T., Kassam, A., Li, H., 2010. Current status of adoption of no-till farming in the world and some of its main benefits. Int. J. Agric. Biol. 3, 1-25.

Drijber, R.A., Doran, J.W., Parkhurst, A.M., Lyon, D.J., 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biol. Biochem. 32,

1419-1430.Engel, F., Bertol, I., Ritter, S., Gonzalez, A.P., Paz-Ferreiro, J., Vazquez, E.V., 2009. Soil erosion under simulated rainfall in relation to phenological stages of soybeans and tillage methods in Lages, SC, Brazil. Soil Till. Res. 103, 216-221.

Garcia, J.P., Wortmann, C.S., Mamo, M., Drijber, R., Tarkalson, D., 2007. One-time tillage of no-till. Agron. J. 99, 1093-1103.

Govaerts, B., Mezzalama, M., Unno, Y., Sayre, K.D., Luna-Guido, M., Vanherck, K., Dendooven, L., Deckers, J., 2007. Influence of tillage, residue management, and crop rotation on soil microbial biomass and catabolic diversity. Appl. Soil Ecol. 37, 18-30.

Green, V., Stott, D., Diack, M., 2006. Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. Soil Biol. Biochem. 38, 693-701.

Hobbs, P.R., Sayre, K., Gupta, R., 2008. The role of conservation agriculture in sustainable agriculture. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 543-555.

Isbell, R., 2002. The Australian soil classification. CSIRO Publishing, Collingwood, Vic, Australia.

IUSS Working Group WRB (2007) 'World reference base for soil resources 2006' World Soil Resources Report No. 103. ftp://ftp.fao.org/docrep/fao/009/a0510e/a0510e00.pdf Accessed on 20th November, 2014.

Joergensen, R.G., Brookes, P.C., 1990. Ninhydrin-reactive nitrogen measurements of microbial biomass in 0.5 m K_2SO_4 soil extracts. Soil Biol. Biochem. 22, 1023-1027.

Kibblewhite, M., Ritz, K., Swift, M., 2008. Soil health in agricultural systems. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 685-701.

Llewellyn, R.S., D'Emden, F.H., 2010. Adoption of no-till cropping practices in Australian grain growing regions. Australian Government, Grains Research and Development Corporation.
http://www.grdc.com.au/uploads/documents/GRDC_adoption_of_no-till.pdf

Llewellyn, R.S., Lindner, R.K., Pannell, D.J., Powles, S.B., 2002. Resistance and the herbicide resource: perceptions of Western Australian grain growers. Crop. Prot. 21, 1067-1075.

López-Garrido, R., Madejón, E., Murillo, J.M., Moreno, F., 2011. Soil quality alteration by mouldboard ploughing in a commercial farm devoted to no-tillage under Mediterranean conditions. Agr. Ecosyst. Environ. 140, 182-190.

Madejón, E., Murillo, J., Moreno, F., López, M., Arrue, J., Alvaro-Fuentes, J., Cantero, C., 2009. Effect of long-term conservation tillage on soil biochemical properties in Mediterranean Spanish areas. Soil Till. Res. 105, 55-62.

Mallarino, A.P., Bordoli, J.M., Borges, R., 1999. Phosphorus and potassium placement effects on early growth and nutrient uptake of no-till corn and relationships with grain yield. Agron. J. 91, 37-45.

Mathew, R.P., Feng, Y., Githinji, L., Ankumah, R., Balkcom, K.S., 2012. Impact of no-tillage and

conventional tillage systems on soil microbial communities. Appl. Environ. Soil Sci. Volume 2012 (2012), Article ID 548620

Mayrberger, J.M., 2011. Studies of genera cytophaga-flavobacterium in context of the soil carbon cycle. Ph.D. Thesis, Michigan State University. Available:

http://etd.lib.msu.edu/islandora/object/etd%3A1028/datastream/OBJ/view. Accessed 28 August 2014.

Melero, S., Panettieri, M., Madejon, E., Macpherson, H.G., Moreno, F., Murillo, J., 2011. Implementation of chiselling and mouldboard ploughing in soil after 8 years of no-till management in SW, Spain: Effect on soil quality. Soil Till. Res. 112, 107-113.

Nannipieri, P., Giagnoni, L., Renella, G., Puglisi, E., Ceccanti, B., Masciandaro, G., Fornasier, F., Moscatelli, M.C., Marinari, S., 2012. Soil enzymology: classical and molecular approaches. Biol. Fertil. Soils 48, 743-762.

Page, K., Dang, Y., Dalal, R., 2013. Impacts of conservation tillage on soil quality, including soil-borne crop diseases, with a focus on semi-arid grain cropping systems. Australasian Plant Pathol. 42, 363-377.

Quincke, J., Wortmann, C., Mamo, M., Franti, T., Drijber, R., Garcia, J., 2007. One-time tillage of no-till systems. Agron. J. 99, 1104-1110.

Raper, R., 2002. The influence of implement type, tillage depth, and tillage timing on residue burial. T. ASAE 45, 1281-1286.

Roldán, A., Salinas-García, J., Alguacil, M., Díaz, E., Caravaca, F., 2005. Soil enzyme activities suggest advantages of conservation tillage practices in sorghum cultivation under subtropical conditions. Geoderma 129, 178-185.

Schellenberger, S., Drake, H.L., Kolb, S., 2011. Functionally redundant cellobiose-degrading soil bacteria respond differentially to oxygen. Appl. Environ. Microb. 77, 6043-6048.

Sharma, M., 2014. Actinomycetes: Source, identification, and their applications. Int. J. Curr. Microbiol. App. Sci 3, 801-832.

Sharma, P., Singh, G., Singh, R.P., 2013. Conservation tillage and optimal water supply enhance microbial enzyme (glucosidase, urease and phosphatase) activities in fields under wheat cultivation during various nitrogen management practices. Arch. Agron. Soil Sci. 59, 911-928.

Thomas, F., Hehemann, J.-H., Rebuffet, E., Czjzek, M., Michel, G., 2011. Environmental and gut bacteroidetes: the food connection. Front. Microbiol. 2, article 93.

Thomas, G.A., Titmarsh, G.W., Freebairn, D.M., Radford, B.J., 2007. No-tillage and conservation farming practices in grain growing areas of Queensland – a review of 40 years of development. Aust. J. Exp. Agr. 47, 887-898.

Wardle, D., 1992. A comparative assessment of factors which influence microbial biomass carbon

and nitrogen levels in soil. Biol. Rev. 67, 321-358.

Wortmann, C.S., Drijber, R.A., Franti, T.G., 2010. One-time tillage of no-till crop land five years post-tillage. Agron. J. 102, 1302-1307.

Wortmann, C.S., Quincke, J.A., Drijber, R.A., Mamo, M., Franti, T., 2008. Soil microbial community change and recovery after one-time tillage of continuous no-till. Agron. J. 100, 1681-1686.

Supplementary information is associated with the online version of this paper

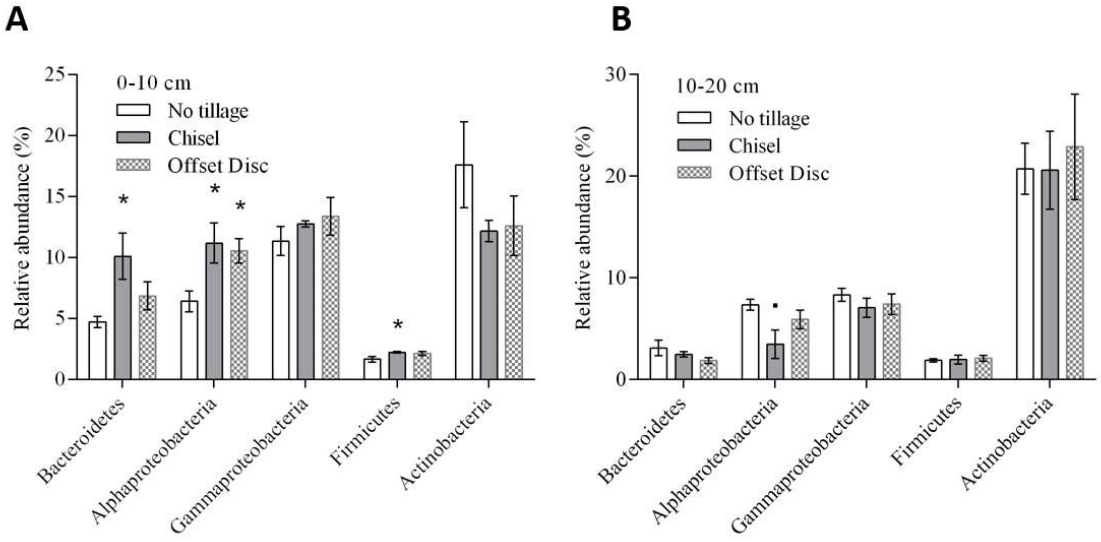

Fig. S1 The relative abundances of bacterial groups associated with NT and tilled soils. Error bars represent standard deviations of the mean and the asterisks/dots indicate differences between treatments and the NT ($P < 0.1$ (.), $P < 0.05$ (*), LSD).

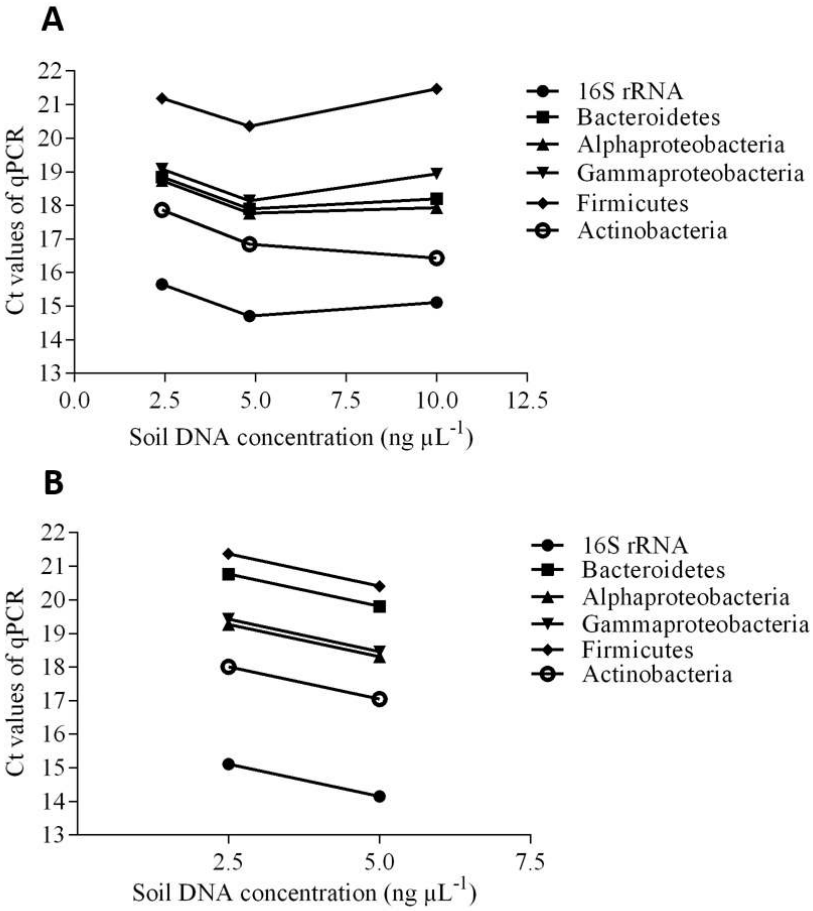

Fig. S2 Inhibition tests for qPCR used for profiling the compositions of soil bacterial communities. The Ct values of qPCR were plotted versus the DNA concentration at 0-10 cm soil depth (**A**), and at 10-20 cm soil depth (**B**). No significant inhibition effects by using 5 ng μL^{-1} of soil DNA on qPCR were observed.

Chapter 3 Strategic tillage on a Grey Vertosol after fifteen years of no-till management had no short-term impact on soil properties and agronomic productivity

Overview

In this chapter, we continued to determine the critical aspects of ST regarding its influences on the long-term NT soils. Timing and implement type of ST are important determinants for the successful tillage operations in the NT fields. This chapter provides insight into when and how ST operation is implemented in an otherwise NT systems to minimise its impacts when combating the constraints of the NT farming systems. The effects of ST using different timing and implement on soil physicochemical and biological properties as well as agronomic productivity were tested on a 15-year continious NT grey Vertosol in Moree, Australia.

Highlights

- Strategic tillage was applied on a grey Vertisol with fifteen years of NT in Australia;
- Strategic had no short term impact on soil properties or agronomic productivity;
- Strategic tillage has great potential to assist in weed management.

Strategic tillage on a Grey Vertosol after fifteen years of no-till management had no short-term impact on soil properties and agronomic productivity

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Abstract

Over half of the arable land in the northern grains region of Australia is managed using no-till (NT), a farming method which has improved crop yields and soil quality while reducing the input and labour costs. However, concerns have arisen among farmers over the control of weeds in continuous NT systems. An occasional targeted tillage operation (termed strategic tillage - ST) has been proposed as a management tool to reduce problem weed populations but may adversely influence soil properties and those of associated microbial communities. To assess the potential impacts of a ST operation on soil properties, a Grey Vertosol with fifteen years of NT in Northern New South Wales, Australia was tilled using either a chisel cultivator or disc chain on March 15th 2013 or on April 5th 2013. We hypothesised that ST using these minimal or low soil inversion implements at either timing would not adversely influence soil properties in the short-term (4-7 weeks). The measured soil properties were soil volumetric moisture content (VMC), pH, bulk density (BD), electrical conductivity (EC), available phosphorus (P), soil organic carbon (SOC), microbial biomass carbon (MBC), metabolic activity (MA), genetic structures of bacterial communities and wheat yield (t ha^{-1}). We found that ST with either a chisel cultivator or a disc chain has great potential to assist in weed management as it did not statistically influence crop productivity or the physical, chemical and biological properties of the soil, regardless of the tillage timing.

Key words

MicroResp^{TM}; T-RFLP; wheat; one-time tillage; weed control; soil bacterial communities

Abbreviations

CCM: chisel cultivator on March 15th, 2013; DCM: disc chain on March 15th, 2013; CCA: chisel cultivator on April 5th, 2013; DCA: disc chain on April 5th, 2013.

1 Introduction

No-till (NT), as a sustainable agricultural practice, has experienced an increasing rate of adoption during the last decade, and is currently being practiced on over 125 million hectares of arable land worldwide (Friedrich et al., 2012). Within Australia in 2014, NT farm management was employed on 17 million hectares, accounting for 13.6% of the world's NT cropping lands (FAO, 2014). NT has led to many benefits in cost-effectiveness (less fuel and labour), crop productivity (increase in crop yield) and environmental improvements, such as increased organic carbon, reduction in soil erosion and increases in soil biological biodiversity (Bayer et al., 2006; Dang et al., 2015b; Triplett and Dick, 2008).

However, long-term NT soils are prone to problems such as soil compaction, nutrient stratification in surface layers of the soil profile, stubble- or soil-borne diseases and prevalence of herbicide-resistant weeds (Dang et al., 2015b). In Australia, herbicide-resistant weeds have become a major threat to agricultural productivity. A survey carried out in 2008 by the Australian Grains Research and Development Corporation indicated that widespread herbicide-resistant weeds along with the increased price of herbicides has led many farmers to apply occasional tillage operations to combat weeds in their NT farms (Llewellyn and D'Emden, 2010). Strategic tillage (ST), which refers to the practice of occasional tillage utilising a variety of implements and timings, may address these problems without compromising the benefits of NT. Yet, tillage in any form will inevitably change the soil physical, chemical properties and the habitats of soil biota. It is this change and the impact on productivity that needs to be assessed to fully understand the risks associated with ST.

The impact of ST largely depends on the tillage implement used. For instance, tillage with a mouldboard plough (MP) is reported to cause greater impacts on soil properties as compared with chisel or disc (Dang et al., 2015b). However, even destructive ST operations with a MP have produced variable results. Either negative and positive impacts (Grandy and Robertson, 2006; López-Garrido et al., 2011; Melero et al., 2011; Pierce et al., 1994) or no changes (Kettler et al., 2000; Wortmann et al., 2010) have been reported from the imposition of ST on soil quality and productivity. A common theme of the aforementioned studies was the use of a MP, which is representative of industry standards for the trial locations within America and Europe. Implements which cause less/minimal soil inversion such as disc, tine and chisel tillage are commonly used in

the northern grain-growing regions (NGR) of Australia (Dang et al., 2015b). The impacts on the physical and chemical soil properties and especially the change in habitats of soil biota regarding the use of these implements for ST are largely unknown.

To address this, a base level of information is required on soil microbial biomass carbon (MBC), microbial activity and genetic structure of the microbial communities in different soil types and climatic conditions. Recent reviews by Dang et al. (2015a; 2015b) and research by Crawford et al. (2015) and Liu et al. (2015) have begun to explore the possible impacts of ST in NT systems in different soil types and climatic regions. Crawford et al. (2015) stated that soil total microbial activity (TMA) was not affected by ST when utilising less/minimal soil inversion implements. This study however, did not explore the tillage effects on soil MBC and the composition of bacterial communities, and therefore knowledge gaps need to be explored to better understand the impacts on different soil types.

The primary aim of this study was to identify possible impacts of timing and the type of tillage implement used in a ST on a long-term NT farm with regards to soil productivity, physical, chemical and biological properties. Based on the fact that disc chain and chisel cultivator are tillage implements that produce minimal soil inversion compared to a MP, our hypothesis is that ST using these two implements would not change soil properties and agronomic productivity even in the short-term. In conjunction with the widely used soil parameters of volumetric moisture (VCM), pH, bulk density (BD), electrical conductivity (EC), available P, and total soil organic carbon (SOC), a suite of biological indicators including MBC, metabolic activity (MA), total microbial activity (TMA) and soil bacterial genetic fingerprinting were used to test this hypothesis. The method used for measuring soil MA in this study was MicroResp^{TM} analysis which is a cheap but quick and effective method through assessing soil carbon substrate utilization ability (Campbell et al., 2003). Quantitative real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) were used for determining the structure of soil bacterial communities, and the latter method was demonstrated to be as a robust and reproducible method as pyrotag sequencing in covering integrate bacterial communities in soils (Pilloni et al. 2012). Altogether, our approach of using the selected soil indicators and methods is predicted to be powerful for discriminating between soil properties from different ST treatments.

2 Materials and Methods

2.1 Site description

The experimental field selected for this study was located approximately 65 km North East of Moree, New South Wales, Australia (29°08'S, 150°07'E). The soil was an Endocalcareous Epipedal Grey Vertosol (Australian Soil Classification (ASC), Isbell 2002) or Vertisol (World Reference Base (WRB), IUSS 2006) developed on Croppa Creek Plains: extensive alluvial fans and rolling downs on Quaternary sediments and planar surfaces of Cretaceous calcareous silty sandstones and shales (Isbell, 2002; Németh et al., 2002). The mean annual precipitation is 610 mm, and the mean annual maximum and minimum temperature ranged between 12.2°C and 26.5°C. The rainfall history is shown in Fig. 1. A summary of chemical and physical properties of Moree soils is described in Table 1. The experimental site has been under NT management for fifteen years, with the most recent crop grown immediately before collection of soil samples being chickpea (*Cicer arietinum* L.). Common weed species at the experimental site were African Turnip (*Sisymbrium thellungii*), Milk Thistle (*Sonchus oleraceus*), Scotch Thistle (*Cirsium vulgare*) and Wild Oats (*Avena fatua*).

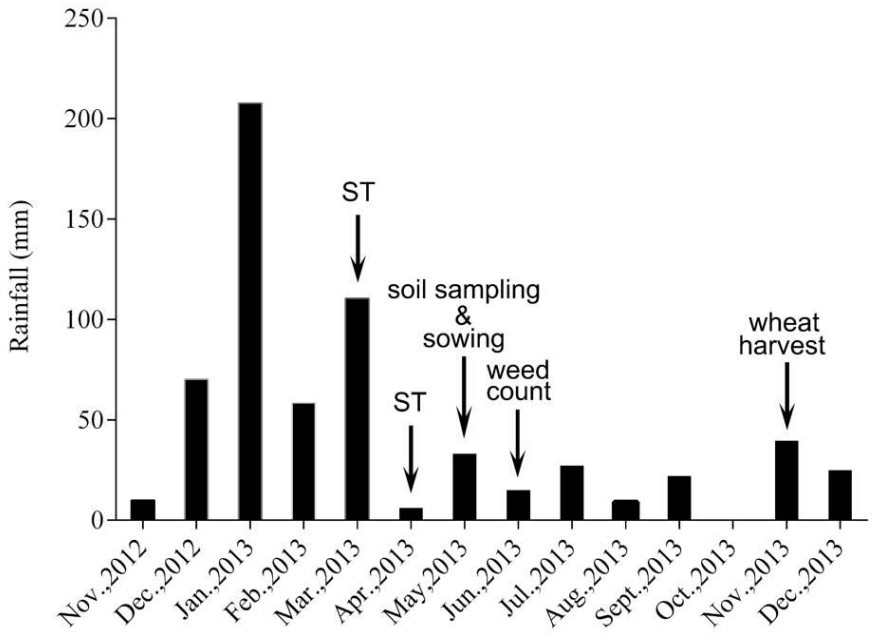


Fig. 1 Rainfall conditions recorded from November, 2012 to December, 2013 at the experimental site (obtained at http://www.bom.gov.au/).

Table 1 Physicochemical properties at 0-10 cm and 10-20 cm soil depths.

	TN	Al	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn	clay	silt	sand
Depth (cm)	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
0-10	0.89	1.93	1466	5.76	1.35	1252	365	980	550	78	366	27	310	120	570
10-20	0.54	2.79	2565	6.43	1.96	1052	664	1092	927	113	251	28	390	110	500

Note: TN represents total nitrogen. TN was measured according to handbook section: 6B2, p75 (Rayment and Lyons, 2011); Soil total mineral was determined by USEPA method 3052, titled "Microwave assisted acid digestion of siliceous and organically based matrices", Kingston HM and Walter PJ (Rayment and Lyons, 2011).

2.2 Experimental design and sampling protocol

The experimental design was a randomised complete block (12 m × 100 m) with four replications per treatment. A single ST with either a chisel cultivator or a disc chain was applied within the farm management spray regime on March 15th, 2013 and April 5th, 2013 instead of herbicide treatment to a depth of 0-10 cm (disc chain) or 0-15 cm (chisel cultivator). The effect of two factors was examined in this study: I) chisel cultivator or disc chain as tillage implements; II) March 15th or April 5th for application timing. Soil chemical and physical properties were analysed at depths of 0-5 cm, 5-10 cm, 10-20 cm and 20-30 cm; and soil microbial properties were analysed at depths of 0-10 cm and 10-20 cm. The soil health indicators investigated in this study were soil volumetric moisture content (VMC), pH, bulk density (BD), electrical conductivity (EC), available phosphorus (P), soil organic carbon (SOC), microbial biomass carbon (MBC), metabolic activity (MA), compositions of bacterial communities and soil agronomic productivity. Soil agronomic productivity was assessed by wheat (*Triticum aestivum* L.) grain yield (t ha^{-1}) for the 2013 winter cropping season.

Soil samples for physical and chemical analysis were collected on the 3rd of May 2013 to depths of 0-30 cm using a tube sampler (43 mm in diameter) attached to a hydraulic soil sampling rig. Two soil samples were taken in each plot and were sectioned into depths of 0-5 cm, 5-10 cm, 10-20 cm and 20-30 cm. Seven sub-soil samples were taken from both 0-10 cm and 10-20 cm depths using a hand shovel at each plot to be used for soil microbial analysis. All samples collected from the same depth of the same plot were mixed thoroughly after sampling on site. Soils were then transported to the laboratory where they were sieved (porosity < 4 mm) and tested for gravimetric water content immediately, before they were stored at 4°C until used for further tests.

2.3 Physical and chemical analysis

Bulk density was calculated from the first sample by taking the mass of oven-dried soil (105^{o}C) per unit volume of the soil sample. The calculation of VMC involved multiplying the gravimetric moisture content with the BD value. To determine soil EC and pH, the second sample was used. The process involved 20 g oven-dry (48 hours at 40°C) soil and pH/EC aqueous (1:5) electrode (Method 3A1 & 4A1, Rayment and Lyons 2011). The Colwell procedure was used to determine available P, and SOC was determined using the method previously developed by Rayment and Lyons (2011).

2.4 Microbial biomass carbon (MBC)

The fumigation-extraction method was used to estimate total soil MBC (Joergensen and Brookes, 1990). MBC was calculated as the difference between organic carbon (C) of fumigated soils and organic C of non-fumigated soils divided by the constant soil-specific calibration coefficient kEC of 0.45.

2.5 Microbial activity assays

2.5.1 Utilisation of carbon substrates

Community-level physiological profiles were determined by the multi substrate-induced respiration (SIR) approach using the MicroResp^{TM} system (James Hutton Institute, Invergowrie, Scotland, UK) (Campbell et al., 2003). A total of fifteen pre-dispensed C sources were used as C substrates, including carboxylic acids (citric acid, L-malic acid, methyl pyruvate, oxalic acid, D+galacturonic acid), amino acids (L-arginine, L-alanine, γ-aminobutyric acid), carbohydrates (β-d-fructose, D-(+)- trehalose, D- glucose, D- xylose, D+ cellubiose, mannitol), and one polymer (tween 80), with Milli-Q water used as a control. To prepare soil samples, 0.38-0.50 g of soil was placed at the bottom of a deep-well plate. The moisture of all soil samples was adjusted to 30% by adding milli-Q water into each deep well. Soil samples were incubated at 25°C for 3 days in a sealed plastic box containing a dish of self-indicating soda lime. Either 7.5 mg or 30 mg C substrate per gram soil water was added into each deep well according to Liu et al. (2015), and tri-replicates were used for each carbon substrate. The assembled MicroResp^{TM} system was incubated at 25°C for 6 hours. The well colour development on detection microplates after incubation was measured at 570 nm. The CO_2 production rate (μg CO_2-C g^{-1} h^{-1}) was subsequently calculated from the difference between the absorbance at 6 h and the 0 h reading before exposure.

2.5.2 Total microbial activity (TMA)

The fluorescein diacetate (FDA) assay was performed to determine soil TMA according to Green et al. (2006). A 15 mL aliquot of 60 mM potassium phosphate buffer (pH 7.6) was added to two grams of soil in a sterile Falcon tube. To start the reaction, 200 µL of 2 mg mL^{-1} FDA solution was added as substrate and shaken at 150 rpm, 30°C for 1 h. To stop the reaction, 950 µL was taken from each reaction and mixed thoroughly with an equal volume of 2:1 (v: v) chloroform: methanol. The obtained mixture was subsequently centrifuged at 12,000 g for 3 min, and 250 µL of the supernatant was transferred into 96 well-plates and the absorbance was measured at 450 nm in a microtiter plate reader (BMG Lab, Ortenberg, Germany).

2.6 Composition of bacterial community

2.6.1 Quantification of bacterial groups

Soil genomic DNA was extracted by combining a sonication step prior to using a commercially available PowerSoil® DNA isolation Kit (MOBIO Laboratories, CA, USA) (Liu et al., 2015) and the DNA concentration was measured with a Qubit™ fluorometer by using Quant-iT dsDNA HS Assay Kits (Invitrogen). ST effects on the soil bacterial groups of Bacteroidetes, Alphaproteobacteria, Gammaproteobacteria, Firmicutes and Actinobacteria were determined by using the taxon-specific quantitative polymerase chain reaction (qPCR) protocol as previously described (Bacchetti De Gregoris et al., 2011). All qPCR reactions were performed using a LightCycler® 96 System (Roche Life Science) and contained 1×Faststart SYBR green mix (Roche Diagnostics Ltd.), 3 µL of each primer (0.3 µM), and 2.5 ng of DNA template. PCR conditions were 10 min at 95°C; 30 cycles of 15 s at 95°C, 15 s at 61°C, and 20 s at 72°C, followed by a final elongation step of 5 s at 72°C. The melting curve was determined by adding a cycle of 10 s at 95°C, 60 s at 65°C and 1 s at 97°C. The obtained qPCR data were subsequently analysed by the LightCycler® 96 software.

2.6.2 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

For T-RFLP, amplification of the 16S rRNA gene was performed with universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') labelled with fluorophore 6-FAM at the 5' end and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3'). Reactions were carried out in a solution containing 14.75 µL of ultra-pure water, 5 µL of 5×Phire buffer, 1.25 µL of dNTPs (10 µM), 1.25 µL of a 10 µM fluorescently-labelled 27F, 1.25 µL of a 1492R (10 µM), 0.5 µL Phire® hot- start II,

and 1 μL of DNA template (around 5 ng). The thermal profile used for the amplification of the 16S rRNA was as follows: 30 s at 98°C; 29 cycles of 10 s at 98°C, 30 s at 56°C (annealing) and 45 s at 72°C; 7 min at 72°C for the final elongation step. Two PCR reactions for each DNA sample were performed to prevent amplification biases. The PCR products were firstly examined by electrophoresis on a 1% agarose gel and then were combined. A 20 μL aliquot of combined PCR products was digested by incubating with 1 U of *Msp*I for 2 h. The digested PCR product was further purified by a commercial PCR cleaning kit (Wizard® SV Gel and PCR Clean-Up System, Promega). All digested PCR products were adjusted to the same concentration by using the Qubit™ fluorometer before being sent to AGRF (Australian Genome Research Facility Ltd., Melbourne) for fragment analysis using capillary electrophoresis (AB3730 DNA analyser).

2.7 Weed and wheat productivity assessment

Total weed populations were determined on the 21st of June, 2013 at the wheat tillering growth stage using a 1 m x 1 m quadrat. Four randomly placed quadrats/samples were counted in each plot to account for possible site variability. Harvest was conducted during the month of November 2013 using on-farm machinery and yield mapping.

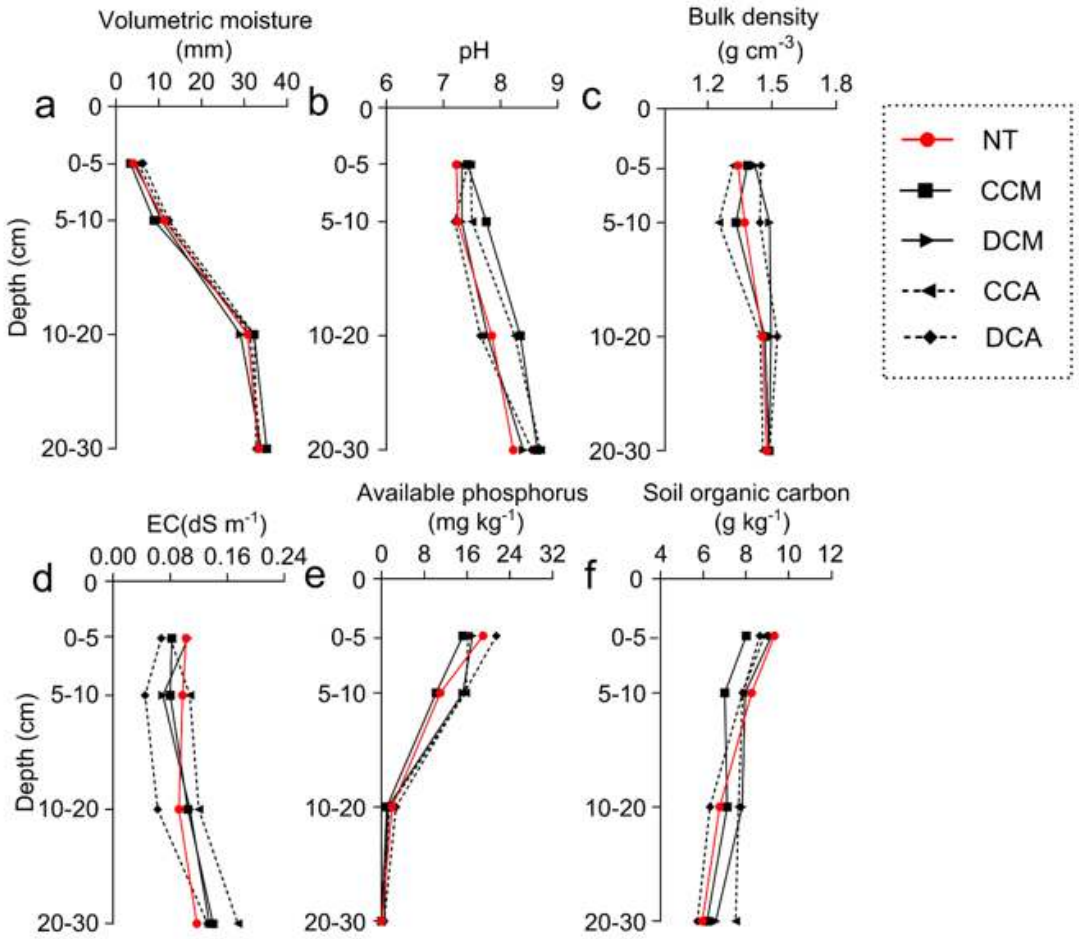
2.8 Statistical analysis

Tillage and depth effects on VMC, pH, BD, EC, available P, SOC, MBC, TMA, utilisation of individual substrates and the wheat yield were examined using ANOVA with post hoc comparison of means using the Least Significant Difference (LSD). The tillage and depth effects on substrate utilisation patterns and the compositions of bacterial communities were examined using PERMANOVA. Prior to ANOA analyses, values of all parameters were tested for normality and homoscedasticity. The soil data of VMC, pH, BD, EC, available P, SOC, MBC, TMA, and qPCR met normality and homoscedasticity assumptions. Z-score and square-root transformation were used for MicroResp and T-RFLP data, respectively, to meet normality and homoscedasticity requirements. Differences in the composition of microbial communities or the utilisation of substrates between samples were visualised using principal component analysis (PCA) and/or heatmaps.

3 Results

3.1 Soil physical and chemical properties

Irrespective of implement or timing used, ST did not overly impact on any soil physical or chemical parameters, including VMC, pH, BD, EC, P and SOC (Fig. 2 a-f). However, these parameters were significantly different between depths except that of BD (one-way ANOVA, $P < 0.05$) (Fig. 2). The VMC ranged from 3.4-6.4 mm (0-5 cm), 8.9-12.4 mm (5-10 cm) in the top soil, and 28.7-32.3 mm (10-20 cm), 31.1-35.2 mm (20-30 cm) in the subsoil. Surface variability was observed in the VMC of the soil tilled by disc chain on April 5th (DCA), which was greater than NT (+35.8%, 0-5 cm, $P = 0.024$). The site had neutral pH in the top soil (pH 7.0-7.5) and was slightly alkaline in the subsoil (pH 7.5-8.5). Bulk density ranged from 1.32-1.45 g cm^{-3} (0-5 cm), being 1.25-1.49 g cm^{-3} (5-10 cm) in the top soil and 1.45-1.53 g cm^{-3} (10-20 cm), 1.46-1.51 g cm^{-3} (20-30 cm) in the subsoil. The EC values ranged from 0.04-0.18 dS m^{-1} in the 0-30 cm. Surface variability was also observed in EC values of the soils tilled by disc chain on April 5th (DCA) which was lower than NT (-51.85%, 0-5 cm, $P = 0.031$), and tillage with DCA lower than disc chain on March 15th (DCM) (-35.71%, 0-5 cm, $P = 0.023$). Available P ranged from 9.5-21.5 mg kg^{-1} in the topsoil and 0-2.75 mg kg^{-1} in the subsoil. Soil organic carbon was 7.0-9.3 g kg^{-1} in the topsoil and 5.7-7.8 g kg^{-1} in the subsoil.



depth (cm)	VMC	pH	BD	EC	available P	SOC
0-5/5-10	***	ns	ns	ns	.	ns
0-5/10-20	***	***	ns	ns	***	**
0-5/20-30	***	***	ns	***	***	***
5-10/10-20	***	**	ns	ns	***	ns
5-10/20-30	***	***	ns	***	***	*
10-20/20-30	**	**	ns	**	ns	ns

Fig. 2 Impacts of ST on (**a**) soil volumetric moisture (VMC), (**b**) soil pH, (**c**) soil bulk density (BD), (**d**) soil electrical conductivity (EC), (**e**) soil available phosphorus, and (**f**) soil organic carbon (SOC). Error bars indicate standard deviations (n = 4). The table below these graphs displays the differences between depths ($P > 0.1$ (ns), $P < 0.1$ (.), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)). NT: no tillage; CCM: chisel cultivator on March 15th, 2013; DCM: disc chain on March 15th, 2013; CCA: chisel cultivator on April 5th, 2013; DCA: disc chain on April 5th, 2013**.**

3.2 Soil biological properties

3.2.1 Microbial biomass carbon (MBC)

In general, the MBC corresponded to 1.17% and 0.59% of the total SOC at depths of 0-10 cm and 10-20 cm, respectively. No significant effect of ST on soil MBC was detected, but the differences between depths (0-10 cm and 10-20 cm) were evident ($P < 0.001$, Table 2).

Table 2 Impacts of ST on MBC (mg C g dry soil^{-1}) and FDA hydrolysis rate (fluorescein μg mL^{-1} g $^{-1}$ soil h^{-1}). Errors represent standard deviations (n = 4). Small and large case letters show significant differences between tillage treatments and soil depths, respectively.

	Depth	NT	CCM	DCM	CCA	DCA
MBC	0-10 cm	0.10±0.02^{aA}	0.11±0.00^{aA}	0.11±0.01^{aA}	0.12±0.01^{aA}	0.13±0.03^{aA}
	10-20 cm	0.04±0.02^{aB}	0.04±0.01^{aB}	0.04±0.00^{aB}	0.03±0.01^{aB}	0.02±0.01^{aB}
FDA	0-10 cm	0.67±0.06^{aA}	0.60±0.03^{aA}	0.76±0.11^{aA}	0.69±0.12^{aA}	0.76±0.06^{aA}
	10-20 cm	0.44±0.10^{aB}	0.36±0.07^{aB}	0.35±0.04^{aB}	0.46±0.06^{aB}	0.41±0.03^{aB}

NT: no tillage; CCM: chisel cultivator on March 15th, 2013; DCM: disc chain on March 15th, 2013; CCA: chisel cultivator on April 5th, 2013; DCA: disc chain on April 5th, 2013

3.2.2 Microbial activity

MicroResp^{TM} physiological profiles were analysed by PCA as shown in Fig. 3 A. The PC1 and PC2 explained 35.6% and 22.0% of the total variance, respectively. Substrate utilisation patterns were not influenced by tillage treatments (PERMANOVA, $P = 0.62$), but utilisation of substrates was typically greater in the 0-10 cm depth than in the 10-20 cm depth (Fig. 3 A, B; PERMANOVA, $P < 0.001$). The depth effect was present in the carbon substrates of oxalic acid, β-d-fructose, D-(+)-trehalose, D-glucose, L-malic acid, D-xylose, D+cellubiose, L-alanine and mannitol (Fig. 3 B). Soil TMA did not differ between tillage treatments and NT at either soil depth, but differed between depths ($P < 0.001$, Table 2).

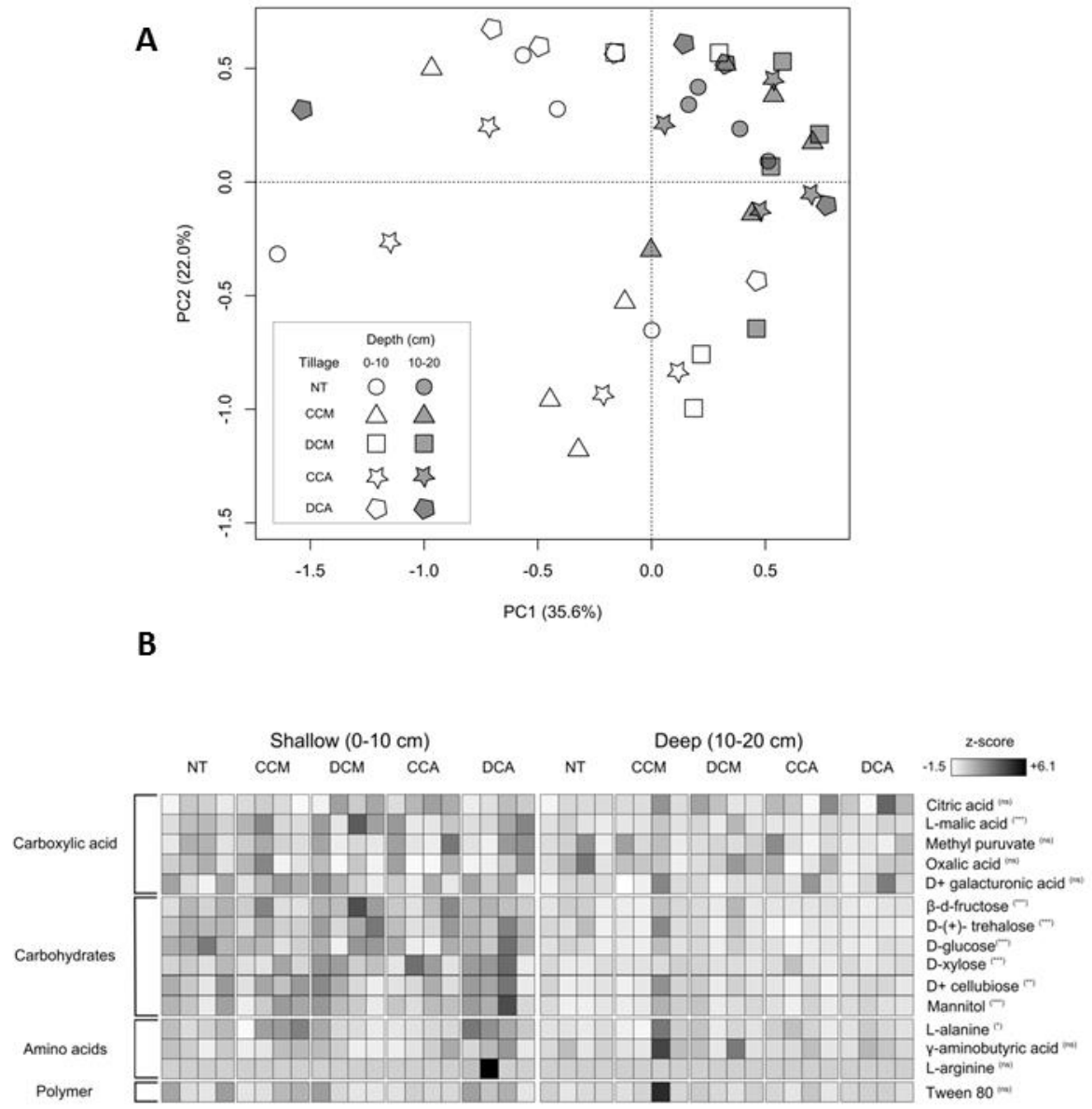


Fig. 3 Principal component analysis (PCA) ordination (A) and heatmap (B) summarising variation in the substrate utilisation profiles between samples based on the z-score transformed C-utilisation (CO_2 evolution) data. Eight carbon substrates, including β-d-fructose, D-(+)-trehalose, D-glucose, L-malic acid, D-xylose, D+cellubiose, L-alanine and mannitol were utilised differentially between depths. The significance of this effect is reflected by the asterisks following each substrate name ($P > 0.05$ $^{(ns)}$, $P < 0.05$ $^{(*)}$, $P < 0.01$ $^{(**)}$, $P < 0.001$ $^{(***)}$).

3.2.3 Composition of bacterial communities

Changes in composition of bacterial communities in response to ST at both soil depths, as assessed by qPCR quantification and T-RFLP, were not detected, irrespective of the tillage

implement and timing used (Fig. 4, Fig 5 A, B). The composition of bacterial communities differed between depths (Fig. 4, Fig 5 A, B; PERMANOVA, $P < 0.001$). Bacterial communities at 0-10 cm depth were associated with larger relative abundances of Actinobacteria, Bacteroidetes, Alphaproteobacteria, while those at 10-20 cm depth were associated with larger relative abundances of Firmicutes and Gammaproteobacteria (Fig. A1 A&B).

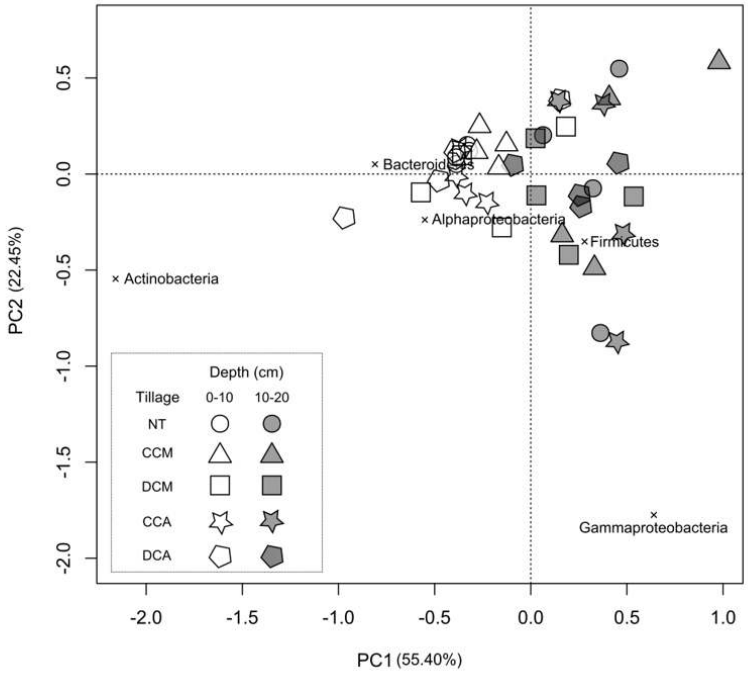


Fig. 4 Principle component analysis (PCA) ordination summarising variation in the composition of bacterial communities between samples based on the qPCR data.

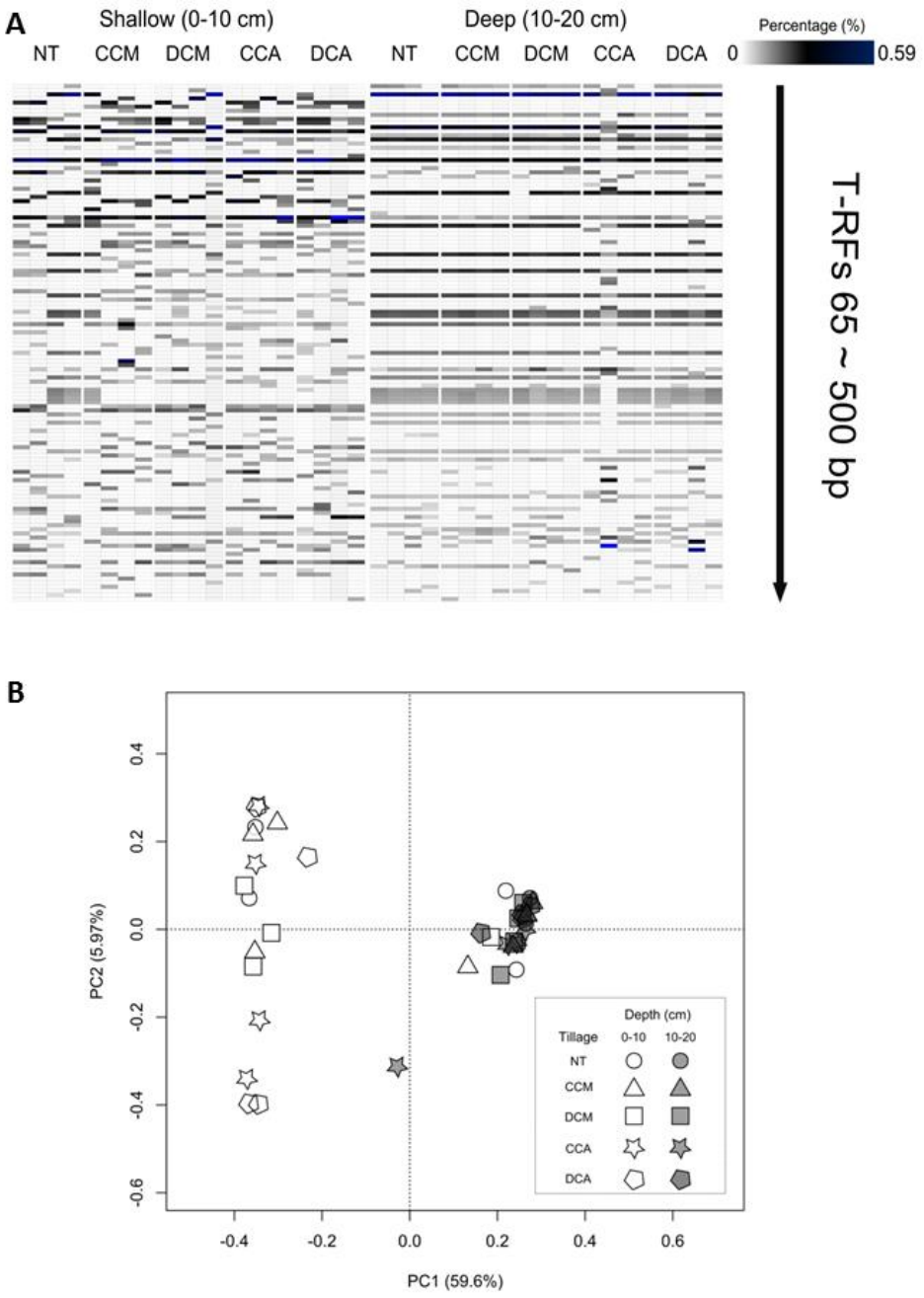


Fig. 5 Heatmap of the frequencies of T-RFs detected in different soil samples. The colour changes from white to green indicate the percentages of each T-RFs changing from 0 to 0.69%. Only those T-RFs ranged between 65 to 500 bp were included in the heatmap and statistical analysis (A); A principle component analysis (PCA) ordination summarising variation in the composition of bacterial communities between samples based on the T-RFLP analysis of full-length 16S rRNA gene amplicons (B).

3.3 Weed and wheat productivity assessment

Weed count was performed on June, 21st, 2013, which was fourteen and seven weeks after the applications of ST. Marginally significant reductions in in-crop weed populations were observed for CCM (68.40%, $P = 0.055$), for DCM (68.40%, $P = 0.055$), for CCA (60.5%, $P = 0.085$), and for DCA (60.5%, $P = 0.085$) fourteen/seven weeks after ST (Table 3). Wheat yields ranged from 3.50-3.63 t ha^{-1} across the treatments. ST did not result in changes of wheat yield compared with NT irrespective of the implements and timing of the tillage operation (Table 3).

Table 3 Impacts of ST on in-crop weed populations (number m^{-2}) and wheat yield (t ha^{-1}). Errors represent standard deviations (n = 4). Weed count was conducted on June, 21st, 2013 (fourteen or eleven weeks after the application of ST).

Tillage treatments	NT	CCM	DCM	CCA	DCA
Weeds	2.36 ± 0.96^a	0.75 ± 0.37^a	0.75 ± 0.10^a	0.94 ± 0.47^a	0.94 ± 0.48^a
Wheat productivity	3.50 ± 0.04^a	3.58 ± 0.05^a	3.51 ± 0.02^a	3.54 ± 0.04^a	3.63 ± 0.06^a

NT: no tillage; CCM: chisel cultivator on March 15th, 2013; DCM: disc chain on March 15th, 2013; CCA: chisel cultivator on April 5th, 2013; DCA: disc chain on April 5th, 2013

4 Discussion

In general, the results of our study support the hypothesis that one-time ST using low/minimal soil inversion implements such as chisel cultivator or disc chain does not influence soil properties and agronomic productivity in the short-term (4-7 weeks). Potential changes to BD and SOC caused by tillage could take a longer time period than the 4-7 weeks' timeframe that this study is focussed on. However, in order to maintain consistency within the microbiological sampling timeframe, BD, VMC and SOC results from 12 months following ST will not be discussed in detail.

4.1 Physical and chemical soil properties

The initial impact of any tillage event regardless of implement type, affects not only the quantity of surface residues but also the effectiveness of weed control, both of which influence soil water storage (Gibson et al., 1992). In the current study, the only influences on soil physical and chemical properties were restricted to the increase of soil VMC along with the decrease of EC in the top 5 cm soil by DCA. EC that measures soil salinity and VMC that measures soil water storage capacity are factors influencing soil properties and plant performance, which have been widely used as indicators for assessing tillage effects on soil health (Schloter et al., 2003). The slight increase of VMC and decrease of EC in soil surface after ST as found in the present study were consistent with

previously reported results (Roldan et al., 2007). While statistically significant, the numerical differences were minor, making it difficult to draw conclusions on the impact of the treatment. This is further emphasised by the overall impacts on VMC and EC, and other soil physical and chemical indicators caused by ST recording very minor changes.

With regards to ST, the key factors driving potential changes in soil properties include: tillage implement, timing, soil type and climate. Minimal soil inversion implements together with low initial VMC are the likely factors influencing the lack of great loss in VMC. Low rainfall between tillage and sampling (Fig.1) and the short sampling timeframe meant that the possible timing differences could not be fully understood. The role of weather patterns and their influence of potential ST impacts were reported in similar research, e.g. Crawford et al. (2015) found that the tillage effects in two Vertosols from similar climates in 2013 did not change significantly among treatments three months after tillage. These Vertosols had a high initial VMC, leading to the conclusion that changes due to tillage in our trial would be unlikely, considering the dry conditions and the initial moisture status. This minor numerical difference was also observed in VMC 12 months after ST after a prolonged dry weather period (results not shown).

The impact of tillage on soil structure is highly dependent on the moisture status of the soil (Dang et al., 2015b). Tillage or traffic on soils with a full moisture profile can lead to issues such as compaction and smearing. As previously mentioned, the VMC was low in our trial site and hence the impacts on BD and other physical structure were unlikely to be changed by a single tillage event, especially after a sustained period of conservation farming practices. There is however, a risk of soil ‘powdering’ which leads to wind erosion and total breakdown of structure in the topsoil if tillage occurs too frequently in dry conditions. Soil BD was not influenced by the tillage treatments. This is consistent with studies on similar soil types (Crawford et al., 2015; Dalal et al., 2011). It was also reported in Crawford et al. (2015) that soil types with texture contrast and weakly structured hard setting soils appear to be most at risk when considering a ST, e.g. Sodosol soils. However, well-structured Vertosols would likely be unchanged. This statement was supported by the 12 month sampling with non-significant differences between treatments observed in BD (results not shown).

The soil pH can influence soil biology, chemical activity and plant growth by affecting the interactions of soil microorganisms, nutrient availability and toxicity within the soil (Karlen et al., 1997). Tillage treatments did not impact on soil pH at all soil depths with the low level of soil inversion a plausible reason for this lack of change. The use of MP in a NT silt loam soil has shown

to increase the soil pH at 0-7.5 cm (Pierce et al., 1994), but utilising MP to fully invert soil to either incorporate clay or bury weed seed banks is rarely used in the current NGR management system. Soil type is another factor influencing the lack of change, as higher clay contents may buffer changes (Packer and Hamilton, 1993). On a brown Sodosol within the same climatic zone as the current trial site, Thomas et al. (2007) stated that soil pH was not affected by tillage or stubble treatments in the 0-10 cm depth. Therefore, it is likely that the combination of relatively high clay content and minimal soil inversion is the reason for the stability of the tested soil properties after ST.

Total organic carbon is a key component of soil, as it affects plant growth, is a trigger for nutrient availability through mineralization and provides a source of energy and nutrients for soil microorganisms (Karlen et al., 1997). No significant effects on SOC were caused by the tillage treatments. It is possible that the incorporation of organic matter by tillage treatment was equal to the rate of decomposition resulting in a steady level of SOC or that the frequency of tillage was not enough to cause changes. The latter is more likely, as the imposition of one-time tillage on long-term NT soils appears to have little effect on soil carbon status, irrespective of the soil types and the implements used (Baan et al., 2009). This result was supported by the 12 month sampling (results not shown).

There was a large concentration of available P found in the soil surface (0-10 cm) relative to the subsoils. Conservation tillage, especially NT, can result in vertical stratification of plant nutrients in the soil profile (Bauer et al., 2002). Nutrient stratification has been extensively depicted in previous studies which documented that not only the biological, but more often physical and chemical properties are stratified in the topsoil of NT (Bergstrom et al., 1998; Cookson et al., 2008; Curci et al., 1997; Madejón et al., 2009; Mathew et al., 2012; Melero et al., 2011; Quincke et al., 2007a; Wright et al., 2005). The grains industry in Australia is one of many potential contributors to the excessive phosphorus (P) and nitrogen (N) concentrations in rivers and lakes (Mathers and Nash, 2009). Studies, such as Vu et al. (2009), Mathers and Nash (2009) and Bünemann et al. (2006), have aimed to measure nutrient stratification of NT soils and the impact of tillage practices with regards to P, but did not include ST in their comparisons. Within the sampling timeframe, available P was not influenced by tillage with chisel or disc implements. This is consistent with Garcia et al. (2007) and Quincke et al. (2007b), who both stated that MP effectively redistributed soil chemical and physical nutrients while one-time disc or chisel did not. As MP is not a tillage implement used regularly in the NGR, addressing nutrient stratification with ST using only chisel and disc implements would most likely be inefficient. A more aggressive tillage approach or the deep

placement of nutrients would be needed to address nutrient stratification. This approach would however require a new environmental risk analysis to be undertaken.

4.2 ST effects on soil biological indicators

4.2.1 Effects of ST on soil microbial biomass carbon (MBC)

The soil MBC is a crucial component for nutrient cycling and energy flow in soil ecosystems, and can be used as an early indicator to monitor soil fertility and quality (Schloter et al., 2003). Application of one-time ST with chisel cultivator or disc chain in this long-term NT Vertosol at two different time points did not result in changes in soil MBC. The minimal soil inversion characteristics of the two implements, low soil moisture status and one time frequency may have contributed to these results. Low water availability can inhibit microbial activity by lowering intracellular water potential and thus reducing hydration and activity of enzymes (Stark and Firestone, 1995). Additionally, long-term NT soils possess greater stability (physical and biological resistance and resilience), which renders the soil with the capability of absorbing disturbance impacts and/or of rapidly recovering (Kuan et al., 2007). For instance, soil microorganisms, such as bacteria and archaea, which have fast growth rates, high degree of physiological flexibility and rapid evolution rates may recover from suppressed conditions very quickly (Allison and Martiny, 2008). It is plausible that soil microorganisms may have recovered to the NT levels during the 4-7 weeks' time after the application of ST, based on the above researches. Our results are consistent with other studies such as López-Garrido et al. (2011); Melero et al. (2011); Wortmann et al. (2008); Wortmann et al. (2010), who reported that chiselling of NT soils did not cause changes in soil MBC.

Many factors including soil management practices, soil type and climate can influence soil microorganisms, which result in a large range of MBC values. In Australian agricultural systems, the normal range of MBC is between 0.02-0.70 mg C g^{-1} soil (Gonzalez-Quiñones et al., 2011). Additionally, Bell et al. (2006) have reported that the MBC in Vertosols under different soil management practices and climates in the NGR of Australia ranged from 0.15 mg C g^{-1} soil to 0.95 mg C g^{-1} soil at 0-5 cm. Therefore, while the MBC determined in our study (around 0.1 mg C g^{-1} soil at 0-10 cm depth and 0.04 mg C g^{-1} soil at 10-20 cm depth) falls within the normal range in Australian agricultural systems, it does, however, indicate a Vertosol of poorer quality in NGR of Australia.

Microbial quotient (Cmic: Corg ratio) reflects the soil ability in converting SOC into MBC (Gonzalez-Quiñones et al., 2011). In general, the microbial quotient was around 1.17% and 0.59% at depths of 0-10 cm and 10-20 cm, respectively. It has been reported that the typical microbial

quotient falls within a relatively narrow range from 0.8% to 4.0% in Australian soils (Gonzalez-Quiñones et al., 2011). The microbial quotient in our study is lower than that reported by Bell et al. (2006), from 2% to 4% for Vertosol soils under different soil management practices and climatic conditions. This may indicate a poor ability in mineralising SOC and potential poor initial crop growth once the soil wets up.

4.2.2 Effects of ST on soil metabolic activity (MA) and total microbial activity (TMA)

TMA, as indicated by the FDA hydrolysis test, covers esterases, lipases and certain proteases (Chaer et al., 2009). Overall, tillage effects on the TMA of the NT Vertosol were not significant, irrespective of the implement types or timing. As a broad-scale method, the FDA hydrolysis assay may not be sensitive enough to evaluate the effect of ST on specific enzymes, such as cellulose and lactase. However, the results of this assay suggested that major soil microbial functions relating to decomposition were maintained in treatments with chisel cultivator and disc chain, regardless of timing used. The FDA hydrolysis assay has been used for determining the impacts of management practices on soil biota in Vertosols in Australian NGRs, where the TMA ranged from 1.2 to 5.4 $\mu g\ mL^{-1}\ g^{-1}$ soil h^{-1} FDA for all soil samples tested (Bell et al., 2006). In comparison, the TMA of the Vertosol in the present study (0.6-0.8 $\mu g\ mL^{-1}\ g^{-1}$ soil h^{-1}, 0-10 cm; 0.3-0.5 $\mu g\ mL^{-1}\ g^{-1}$ soil h^{-1}, 10-20 cm) was much lower. The low soil VMC and SOC most likely contributed to the lower values, but it does warrant further investigation at different moisture status.

Until now, there has been no available information about the utilisation of organic carbon substrates in Australian Vertosols. MA as indicated by the MicroResp™ assay should be another indicator for measuring soil health and quality as it indicates the soil ability in degrading organic substrates and immobilizing nutrients in the microbial biomass (Schloter et al., 2003). The MicroResp™ assay supported the results depicted by the TMA assays. It demonstrated that ST did not influence NT soil efficiency in utilising fifteen carbon sources. This information is vital in understanding how future soil management changes could affect the soil biota and their habitats. The MicroResp™ data in the present study could be used for establishing a future database for soil biological health of Australian Vertosols as it provides information at the lower end of the moisture spectrum.

4.2.3 Effects of ST on genetic structure of soil bacterial community

The effects of ST on the relative abundance of five microbial groups in NT soils were not significant. Further evidence for this was provided by the multivariate data analysis with the T-RFLP fingerprinting. When taking into consideration the level of soil disturbance caused by chisel

cultivator and disc chain, soil resistance/resilience and soil moisture, soil microbial communities of this Vertosol under 15 years NT would likely remain unchanged by tillage treatments. Similarly, Wortmann et al. (2010) have reported that soil microbial community structure in a silty clay loam was not influenced five years after a one-time tillage by MP (Wortmann et al., 2010).

Interestingly, the microbial communities of the long-term NT Vertosol in the current study are dominated by Actinobacteria, which represented almost half of the total soil bacterial communities targeted by the primers used. Mathew et al. (2012) have also reported that Actinobacteria were greatly increased in abundance in a NT system under crop retention. Actinobacteria is a phylum of Gram-positive bacteria, which is crucial for the carbon cycling and nitrogen fixing in agro-ecosystems (Sharma, 2014). Actinobacteria are also drought resistant and have been shown to be able to grow under challenging dry conditions (Barnard et al., 2013). It is uncertain whether the dry soil conditions or the NT management system contributed to the relatively high abundance of this particular phylum, with more research needed on how changing moisture status influences the growth rates in this particular soil type. The implication of this in regard to productivity is largely unknown as the capture of changing soil biota communities with increasing VMC has not been studied in the NGR.

4.3 Weed populations and agronomic productivity

All applications of tillage used in this study caused marginally significant reduction in weed populations. The large variance between replicates may have contributed to the differences being only marginally significant. This result is consistent with Crawford et al. (2015) and Mclean et al. (2012), who also observed a reduction trend in weed populations for similar soil types in Queensland of Australia. Previous use of MP in NT/reduced tillage system always had prominent efficiency in weed control (Douglas and Peltzer, 2004; Kettler et al., 2000; Renton and Flower, 2015). This could be explained by the fact that MP practices bury weed seeds from soil surface (< 1 cm) into deep soil (>15 cm), which stops the emergence of weed seedlings. Compared with MP, a single practice with disc chain and chisel cultivator in our study bury less number of weed seeds to a more even depth throughout soil (Douglas and Peltzer 2004). Therefore, ST with these two implements less efficiently suppressed weed populations in this NT soil is expected. However any reduction in-crop weed populations can be positive for productivity and the longevity of non-invasive weed control as the application of ST could aid in reducing chemical resistance within a NT system. Adoption of continuous NT has contributed to increased use of glyphosate for weed

control and the build-up of herbicide resistant weed populations (e.g. wild oats and African turnip weed as found in this study) (Dang et al 2015a; Werth et al 2008). Providing stakeholders with alternative options to combat in-crop weed populations such as the implementation of ST could assist in future decision making process if the soil quality is maintained. Monitoring the influence of weather patterns is normally possible in longer term trials, whereas in this trial only the short-term changes were assessed. Further research is required regarding ST effectiveness in weed control over the long-term as average weather patterns were not experienced during this study or by Crawford et al. (2015).

Crop yield was not affected by either the tillage treatments or the reduction in weed populations. A plausible conclusion is the climatic conditions of the season during which the weed population was not the main pressure in the experimental site for the 2013 season. Variable results regarding productivity are common in the overall literature concerning tillage. For example, Wortmann et al. (2010) reported that grain yield was not affected by tillage treatment, while a significant positive impact was observed by Kettler et al. (2000) and a negative effect was reported by Diaz-Zorita et al. (2004). No effects have been recently reported on grain yield of NT in the NGR of Australia after one-time tillage using either tine or disc based implements (Crawford et al., 2015). The common denominators with these studies that lead to the variability of the results are different soil types, implements used and climatic conditions. Utilising the correct implement at the right time is dependent on the management system and the weather conditions; if weed pressure is the main concern then a ST could be a viable option on a Vertosol with a similar climate to the site in the current study.

5 Conclusions

Our results indicated that ST could be used as a viable management option within NT farming systems as it causes minimal impacts on soil health and productivity in the NGR of Australia. However, it must be noted that while there were minimal impacts on soil health and a marginally significant reduction of in-crop weed populations, the study timeframe did not allow results within average rainfall weather patterns. Observed significant soil depth differences for all the indicators utilised in this study suggest that they were sensitive enough to detect potential impacts of ST and can act as a guideline for future research. The presence of relative high levels of Actinobacteria in the top soil confirmed the dry soil status during sampling with further research is

required to assess the potential impacts to productivity upon the wetting up process. Further knowledge is also required in the understanding of how soil microbial communities interact within the Australian agricultural systems and in particular the NGR over different timeframes and weather patterns.

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Conflict of interest

The authors declare no conflict of interest.

References

Allison, S.D., Martiny, J.B., 2008. Resistance, resilience, and redundancy in microbial communities. Proc. Natl. Acad. Sci. U.S.A. 105 (Supplement 1), 11512-11519.

Baan, C.D., Grevers, M.C.J., Schoenau, J.J., 2009. Effects of a single cycle of tillage on long-term no-till prairie soils. Can. J. Soil Sci. 89(4), 521-530.

Bacchetti De Gregoris, T., Aldred, N., Clare, A.S., Burgess, J.G., 2011. Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. J. Microbiol. Meth. 86(3), 351-356.

Barnard RL, Osborne CA, Firestone MK (2013) Response of soil bacterial and fungal communtities to extreme desiccation and rewetting. ISME J. **7**, 2229-2241.

Bauer, P.J., Frederick, J.R., Busscher, W.J., 2002. Tillage effect on nutrient stratification in narrow- and wide-row cropping systems. Soil Till. Res. 66(2), 175-182.

Bayer, C., Martin-Neto, L., Mielniczuk, J., Pavinato, A., Dieckow, J., 2006. Carbon sequestration in two Brazilian Cerrado soils under no-till. Soil Till. Res. 86(2), 237-245.

Bell, M., Seymour, N., Stirling, G., Stirling, A., Van Zwieten, L., Vancov, T., Sutton, G., Moody, P.,

2006. Impacts of management on soil biota in Vertosols supporting the broadacre grains industry in northern Australia. Soil Res. 44(4), 433-451.

Bergstrom, D., Monreal, C., King, D., 1998. Sensitivity of soil enzyme activities to conservation practices. Soil Sci. Soc. Am. J. 62(5), 1286-1295.

Bünemann, E.K., Schwenke, G.D., Van Zwieten, L., 2006. Impact of agricultural inputs on soil organisms—a review. Soil Res. 44(4), 379-406.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl. Environ. Microbiol. 69(6), 3593-3599.

Chaer, G., Fernandes, M., Myrold, D., Bottomley, P., 2009. Comparative resistance and resilience of soil microbial communities and enzyme activities in adjacent native forest and agricultural soils. Microb. Ecol. 58(2), 414-424.

Cookson, W.R., Murphy, D.V., Roper, M.M., 2008. Characterizing the relationships between soil organic matter components and microbial function and composition along a tillage disturbance gradient. Soil Biol. Biochem. 40(3), 763-777.

Crawford, M., Rincon-Florez, V., Balzer, A., Dang, Y., Carvalhais, L., Liu, H., Schenk, P., 2015. Changes in the soil quality attributes of continuous no-till farming systems following a strategic tillage. Soil Res. 53(3), 263-273.

Curci, M., Pizzigallo, M.D.R., Crecchio, C., Mininni, R., Ruggiero, P., 1997. Effects of conventional tillage on biochemical properties of soils. Biol. Fertil. Soils 25(1), 1-6.

Dalal, R.C., Allen, D.E., Wang, W.J., Reeves, S., Gibson, I., 2011. Organic carbon and total nitrogen stocks in a Vertisol following 40 years of no-tillage, crop residue retention and nitrogen fertilisation. Soil Till. Res. 112(2), 133-139.

Dang, Y.P., Seymour, N.P., Walker, S.R., Bell, M.J., Freebairn, D.M., 2015a. Strategic tillage in no-till farming systems in Australia's northern grains-growing regions: I. Drivers and implementation. Soil Till. Res. 152, 104-114.

Dang, Y.P., Moody, P.W., Bell, M.J., Seymour, N.P., Dalal, R.C., Freebairn, D.M., Walker, S.R., 2015b. Strategic tillage in no-till farming systems in Australia's northern grains-growing regions: II. Implications for agronomy, soil and environment. Soil Till. Res. 152, 115-123.

Diaz-Zorita, M., Grove, J., Murdock, L., Herbeck, J., Perfect, E., 2004. Soil structural disturbance effects on crop yields and soil properties in a no-till production system. Agron. J. 96(6), 1651-1659.

Douglas, A., Peltzer, S.C., 2004. Managing herbicide resistant annual ryegrass (Lolium rigidum Gaud.) in no-till systems in Western Australia using occasional inversion ploughing,

Proceedings of the Fourteenth Australian Weed Conference, pp. 6-9.

FAO, 2014. http://www.fao.org/ag/ca/6c.html. CA Adoption Worldwide, AQUASTAT data query, ©2014 FAO of the UN, Commissioned for the exclusive use of FAO Conservation Agriculture.

Friedrich, T., Derpsch, R., Kassam, A., 2012. Overview of the global spread of conservation agriculture. FACTS. (Special Issue 6).

Garcia, J.P., Wortmann, C.S., Mamo, M., Drijber, R., Tarkalson, D., 2007. One-time tillage of no-till. Agron. J. 99(4), 1093-1103.

Gibson, G., Radford, B., Nielsen, R., 1992. Fallow management, soil water, plant-available soil nitrogen and grain sorghum production in south west Queensland. Anim. Prod. Sci. 32(4), 473-482.

Gonzalez-Quiñones, V., Stockdale, E., Banning, N., Hoyle, F., Sawada, Y., Wherrett, A., Jones, D., Murphy, D., 2011. Soil microbial biomass—Interpretation and consideration for soil monitoring. Soil Res. 49(4), 287-304.

Grandy, A.S., Robertson, G.P., 2006. Aggregation and organic matter protection following tillage of a previously uncultivated soil. Soil Sci. Soc. Am. J. 70(4), 1398-1406.

Green, V., Stott, D., Diack, M., 2006. Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. Soil Biol. Biochem. 38(4), 693-701.

Isbell R (2002) 'The Australian soil classification.' (CSIRO Publishing, Collingwood, Vic, Australia.)

IUSS Working Group WRB (2006) ‘World reference base for soil resources 2006’ World Soil Resources Report No. 103. ftp://ftp.fao.org/docrep/fao/009/a0510e/a0510e00.pdf Accessed on 20th November, 2014.

Joergensen, R.G., Brookes, P.C., 1990. Ninhydrin-reactive nitrogen measurements of microbial biomass in 0.5 m K_2SO_4 soil extracts. Soil Biol. Biochem. 22(8), 1023-1027.

Karlen, D.L., Mausbach, M.J., Doran, J.W., Cline, R.G., Harris, R.F., Schuman, G.E., 1997. Soil Quality: A Concept, Definition, and Framework for Evaluation (A Guest Editorial). Soil Sci. Soc. Am. J. 61(1), 4-10.

Kettler, T.A., Lyon, D.J., Doran, J.W., Powers, W., Stroup, W.W., 2000. Soil quality assessment after weed-control tillage in a no-till wheat–fallow cropping system. Soil Sci. Soc. Am. J. 64(1), 339-346.

Kuan, H., Hallett, P., Griffiths, B., Gregory, A., Watts, C., Whitmore, A., 2007. The biological and physical stability and resilience of a selection of Scottish soils to stresses. Eur. J. Soil Sci. 58(3), 811-821.

Liu, H., Carvalhais, L., Rincon-Florez, V., Crawford, M., Dang, Y., Paul, D., Schenk, P., 2015. One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till

Calcisol. Soil Till. Res. 10.1016/j.still.2015.12.007. In press.

Llewellyn, R.S., D'Emden, F., 2010. Adoption of no-till cropping practices in Australian grain growing regions. Australian Government, Grains Research and Development Corporation.

López-Garrido, R., Madejón, E., Murillo, J.M., Moreno, F., 2011. Soil quality alteration by mouldboard ploughing in a commercial farm devoted to no-tillage under Mediterranean conditions. Agric. Ecosyst. Environ. 140(1–2), 182-190.

McLean, A.R., Widderick, M.J., Walker, S.R., 2012. Strategic tillage reduces emergence of key sub-tropical weeds, Proceedings of the 18th Australian Weeds Conference. Weed Society of Victoria, Melbourne, Australia, pp. 248.

Madejón, E., Murillo, J., Moreno, F., López, M., Arrue, J., Alvaro-Fuentes, J., Cantero, C., 2009. Effect of long-term conservation tillage on soil biochemical properties in Mediterranean Spanish areas. Soil Till. Res.105(1), 55-62.

Mathers, N.J., Nash, D.M., 2009. Effects of tillage practices on soil and water phosphorus and nitrogen fractions in a Chromosol at Rutherglen in Victoria, Australia. Soil Res. 47(1), 46-59.

Mathew, R.P., Feng, Y., Githinji, L., Ankumah, R., Balkcom, K.S., 2012. Impact of no-tillage and conventional tillage systems on soil microbial communities. App. Environ. Soil Sci. 2012, 10.

Melero, S., Panettieri, M., Madejon, E., Macpherson, H.G., Moreno, F., Murillo, J., 2011. Implementation of chiselling and mouldboard ploughing in soil after 8 years of no-till management in SW, Spain: Effect on soil quality. Soil Till. Res.112(2), 107-113.

Németh M, Janda T, Horváth E, Páldi E, Szalai G (2002) Exogenous salicylic acid increases polyamine content but may decrease drought tolerance in maize. Plant Sci. 162(4), 569-574.

Packer, I.J., Hamilton, G.J., 1993. Soil physical and chemical changes due to tillage and their implications for erosion and productivity. Soil Till. Res. 27(1–4), 327-339.

Pierce, F.J., Fortin, M.-C., Staton, M.J., 1994. Periodic plowing effects on soil properties in a no-till farming system. Soil Sci. Soc. Am. J. 58(6), 1782-1787.

Pilloni, G., Granitsiotis, M.S., Engel, M., Lueders, T., 2012. Testing the limits of 454 pyrotag sequencing: reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes. PLoS ONE 7(7), e40467.

Quincke, J., Wortmann, C., Mamo, M., Franti, T., Drijber, R., Garcia, J., 2007a. One-time tillage of no-till systems. Agron. J. 99(4), 1104-1110.

Quincke, J.A., Wortmann, C.S., Mamo, M., Franti, T., Drijber, R.A., 2007b. Occasional tillage of no-till systems: carbon dioxide flux and changes in total and labile soil organic carbon. Agron. J. 99(4), 1158-1168.

Rayment GE, Lyons DJ (2011) Soil chemical methods-Australasia. CSIRO Publishing,

Collingwood, Victoria, Australia.

Roldan, A., Salinas-Garcia, J., Alguacil, M., Caravaca, F., 2007. Soil sustainability indicators following conservation tillage practices under subtropical maize and bean crops. Soil Till. Res. 93(2), 273-282.

Schloter, M., Dilly, O., Munch, J., 2003. Indicators for evaluating soil quality. Agric. Ecosyst. Environ. 98(1), 255-262.

Sharma, M., 2014. Actinomycetes: Source, identification, and their applications. Int. J. Curr. Microbiol. App. Sci. 3(2), 801-832.

Stark JM, Firestone MK. (1995). Mechanisms for soil moisture effects on activity of nitrifying bacteria. App. Environ. Microbiol. 61, 218-221.

Thomas, G.A., Titmarsh, G.W., Freebairn, D.M., Radford, B.J., 2007. No-tillage and conservation farming practices in grain growing areas of Queensland – a review of 40 years of development. Aust. J. Exp. Agric. 47(8), 887-898.

Triplett, G., Dick, W.A., 2008. No-tillage crop production: a revolution in agriculture! Agron. J. 100 (Supplement_3), S-153-S-165.

Vu, D., Tang, C., Armstrong, R., 2009. Tillage system affects phosphorus form and depth distribution in three contrasting Victorian soils. Soil Res. 47(1), 33-45.

Werth, J.A., Preston, C., Taylor, I.N., Charles, G.W., Roberts, G.N., Baker, J., 2008. Managing the risk of glyphosate resistance in Australian glyphosateresistant cotton production systems. Pest Manag. Sci. 64, 417-421.

Wortmann, C.S., Drijber, R.A., Franti, T.G., 2010. One-time tillage of no-till crop land five years post-tillage. Agron. J. 102(4), 1302-1307.

Wortmann, C.S., Quincke, J.A., Drijber, R.A., Mamo, M., Franti, T., 2008. Soil microbial communites to change and recovery after one-time tillage of continuous no-till. Agron. J. 100(6), 1681-1686.

Wright, A.L., Hons, F.M., Matocha Jr, J.E., 2005. Tillage impacts on microbial biomass and soil carbon and nitrogen dynamics of corn and cotton rotations. App. Soil Ecol. 29(1), 85-92.

Chapter 4 Strategic tillage increased the relative abundance of Acidobacteria but did not impact on overall soil microbial properties of a 19-year no-till Solonetz

Overview

In this chapter, I continue to examine the influence of different frequencies of ST with chisel on soil microbial properties. The effects of one- or two-time tillage events on the soil microbial properties of an acidic Solonetz with 19-year NT management were tested. Traditional parameters, including soil microbial biomass C and N, community-level physiological profiling, total microbial activity and N cycling genes, were measured for soil samples. The composition of soil microbial communities was determined using terminal reaction fragment length polymorphism (T-RFLP) and next generation 16S rRNA Illumina sequencing.

Highlights

- Strategic tillage was applied on a Solonez soil with nineteen years of NT in Australia;
- Strategic tillage had no short term impact on soil microbial properties;
- One-time chisel tillage increased the relative abundance of Acidobacteria RB41 and Acidobacteria iii1-15 at 0-10 cm soil depth of the NT soils;
- Two-time chisel slightly increased the average C utilisation at 10-20 cm soil depth.

Strategic tillage increased the relative abundance of Acidobacteria but did not impact on overall soil microbial properties of a 19-year no-till Solonetz

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Abstract

Continuous no-till (NT) farming is widely practiced in Australia but it is prone to weed infestation. Strategic tillage (ST) can be used to effectively control weeds; however, it is unclear whether ST influences soil microbial properties. We investigated whether one- or two-time tillage events using a chisel plough influence the soil microbial properties of an acidic Solonetz with 19-year NT management. Soil samples were collected from 0-10 and 10-20 cm soil depths, one year post-ST after a chickpea crop. Soil microbial biomass C (MBC) and N (MBN), community-level physiological profiling (CLPP, MicroResp^{TM}) and fluorescein diacetate as an indicator of total microbial activity (TMA) were determined for soil samples. The composition of soil microbial communities was profiled using terminal reaction fragment length polymorphism (T-RFLP) and 16S rRNA sequencing. Detection and DNA-based quantitation of *ChitinaseA*, *nifH*, *amoA*, *narG*, *nirK* and *nosZ* genes were used to assess ST effects on soil C and N cycling potential of the NT via quantitative PCR. Our results show that one- and two-time chisel did not change soil MBC/MBN, TMA or CLPP. Likewise, ST did not change the composition of soil microbial communities and the abundance of genes expressing enzymes involved in key steps of C and N transformations at either soil depth. However, one-time chisel increased relative abundance of Acidobacteria RB41 and Acidobacteria iii1-15, and two-time chisel slightly increased the average C utilisation, both at 10-20 cm soil depth. This suggests that even after a cropping season of chickpea, ST effects on soil microbial properties of the NT Solonetz were negligible. One- and two-time chisel could potentially address the issues associated with long-term NT without impacting overall soil microbial properties.

Key words:

Acidobacteria; Actinobacteria; conservation farming; carbon and nitrogen cycling; soil microbial properties

1 Introduction

In comparison with conventional tillage, no-till (NT) has tangible economic advantages such as potential increase in crop productivity, reduced labour requirements, less energy and machinery inputs (Dang et al. 2015a; Derpsch et al. 2010). Continuous no-till (NT) management alters soil physical and biochemical properties, creating a less disturbed habitat for soil biota than conventional farming (Miura et al. 2015; Sapkota et al. 2012). Environmentally, NT maintains soil moisture, increases soil biodiversity, reduces or eliminates soil erosion and mitigates greenhouse gas (N_2O) emission (Bayer et al. 2006; Engel et al. 2009; Oorts et al. 2007; Triplett and Dick 2008). While NT largely improves soil quality and leads to a more sustainable agriculture compared to conventional practices, concerns have arisen among growers about the excessive use of herbicides and build-up of herbicide-resistant weed populations in Australian NT systems (e.g. ryegrass, *Lolium rigidum* Gaudin) (Walker 2012). Within Australia, some growers have resorted to the use of occasional tillage as an alternative method to address weed issues of the NT (Llewellyn and D'Emden 2010). Strategic tillage (ST) refers to the opportunistic use of occasional tillage in otherwise NT fields by taking into consideration the timing, implement and frequency of the tillage (Dang et al. 2015a). But despite the great potential of weed control with ST, there is a risk that it may undo the NT benefits accumulated over long-term conservation farming.

Any tillage can potentially alter soil physical and chemical profiles as well as the habitats of the microbes, but to what extent this practice influences the NT soil depends on many interacting soil, tillage and climatic conditions (Dang et al. 2015b; Kaurin et al. 2015). The influence of occasional tillage on soil microbial properties has been reported in North America and Europe but results have been inconsistent. López-Garrido et al. (2011) reported that one-time tillage with mouldboard plough (MP) reduced microbial biomass C (MBC), activities of dehydrogenase, β-glucosidase and o-diphenol oxidase in an 11 year NT Eutric Leptosol. In agreement with López-Garrido et al., Melero Sánchez et al. (2011) also found that one-time MP tillage adversely influenced MBC and microbial biomass N (MBN), and activities of dehydrogenase and β-glucosidase of a NT calcareous soil; however one-time chisel did not cause soil changes. In contrast, Wortmann et al. (2010) reported a weaker response of NT soils to one-time MP. The disparity among these studies could be attributed to the difference in soil types, implements used, climates and sampling strategies in each study. Contrarily from Europe and North America, the implements that are commonly used in Australia for tillage are chisel and disc (Dang et al. 2015a). We have previously found that one- or two-time ST with chisel significantly reduced the in-crop weed population (by 84.48%, one-time chisel; by 55.17%, two-time chisel) of a NT Solonetz and

the first year grain of chickpea has been increased in yield (by 8.6%, one-time; by 10.5%, two-time chisel) after ST (Crawford et al. 2015). However, it has not been reported yet whether one- or two-time chisel causes changes in microbial properties of this NT Solonetz.

There are multiple parameters which could be used for assessing soil biological health. Both MBC and MBN are simple methods for assessing the anthropogenic and environmental impacts on soil quality (Sparling et al. 1997). Soil type, soil management practices and climatic conditions are important determinants in controlling soil microbial biomass (Bell et al. 2006; Campbell et al. 1997; Van Gestel et al. 1993). Within any region, the variability between the aforementioned controlling factors underlines the importance of an extensive database to be maintained and developed. In Australian agricultural systems, previous research has recorded the normal range of MBC to be between 0.02 and 0.95 mg C g^{-1} soil (Bell et al. 2006; Gonzalez-Quiñones et al. 2011). The soil type studied by Bell et al. (2006) was a Vertosol. Building on this knowledge can not only strengthen the knowledge of microbial interactions within Australian soils and climates but reduce the uncertainty of minimal sample size which is present currently. Fluorescein diacetate hydrolysis, as an indicator of total microbial activity (TMA), and community-level physiological profiling (CLPP) are parameters typically used to measure soil microbial activity. The commonly used technology for CLPP analyses are the Biolog EcoPlate™-CLPP and Microresp™-CLPP systems. Microresp™-CLPP is a whole-soil method based on substrate-induced respiration which overcomes several drawbacks of the Biolog approach including that of being culture-dependent (Nannipieri et al. 2003). Microresp™-CLPP is also a cost-effective, sensitive and rapid method to assess the functional diversity of microbial communities (Campbell et al. 2003; Nannipieri et al. 2003).

Understanding C and N cycling is crucial for energy flow and nutrient circulation in ecosystems and agricultural systems (Sparling et al. 1997). Soil C and N cycling are however, sometimes overlooked with regards to microbiological interactions, as the techniques to determine them can be challenging and the interactions are hard to quantify. Nitrogen is an essential macronutrient for plants and its availability in the soil has major impacts on crop performance (Haynes 2012). Inproper soil management may not only cause losses of soil fertility but may also result in possible water eutrophication and potential greenhouse gases (Galloway et al. 2004). Analysis of some important soil N and C cycling genes informs whether tillage impacts on the potential functions of the NT soils. This could be a useful complement to the phylogenetic analysis of bacteria in the NT soils. Developing a database for Australian soil regarding soil C and N cycling genes and filling the gaps in the present literature will aid in management decisions. Composition of soil bacterial communities can be profiled by methods of ester-linked and phospholipid-linked fatty

acid methyl esters (FAMFs), quantitative polymerase chain reaction (qPCR), terminal reaction fragment length polymorphism (T-RFLP) and 16S ribosomal RNA (rRNA) sequencing (Kirk et al. 2004). The PCR-based fingerprinting technique of T-RFLP primarily provides population-specific signatures while current technologies on 16S rRNA high throughput sequencing allow phylogenetic identification of microbial populations up to the genus level. Both T-RFLP and 16S rRNA deep sequencing allow analysis of population compositions of complex soil microbial communities (Lee et al. 2011).

In the present study, our aim was to examine the effects of ST on soil biological properties and also to establish the baseline values of various soil parameters for Australian soils. Our hypothesis is that the effects of ST on the composition and activity of the soil microbial communities and N and C cycling potentials of the long-term NT Solonetz after a chickpea crop (*Cicer arietinum*) are small. Parameters including soil MBC and MBN, TMA and MicroResp^{TM}-CLPP were used to assess soil health. We then determined potential effects of ST on soil N and C cycling by quantitating the abundance of a subset of microbial genes involved in C and N cycling using genomic DNA-based qPCR. Furthermore, the composition of soil microbial communities was profiled by T-RFLP and high throughput 16S rRNA amplicon sequencing.

2 Materials and methods

2.1 Site descriptions, tillage treatments and samplings

2.1.1 Site descriptions

The field trial was conducted at Condamine, Queensland in the Northern Grains Region of Australia (26.90°S, 149.64°E). The Condamine soil which is developed on Cainozoic sand plains was classified as mesotrophic effervescent brown Sodosol (Isbell 2002) or Solonetz (IUSS Working Group WRB, 2007). It contains 25% clay, 14% silt and 61% sand on the top surface; 41% clay, 12% silt and 47% sand in the subsoil (Table 1). Condamine receives an annual precipitation of 652 mm, and the mean annual maximum and minimum temperature of this site ranges between 12.2°C and 27.1°C, respectively. The experimental site has a 19-year NT history, and weed infestations of fleabane (*Erigeron annuus*) and feathertop rhodes grass (*Chloris virgata*) have been identified throughout the field trial. Before ST operation in 2012, the previous crop on the experimental field was wheat (*Triticum aestivum*). Chickpea was planted on the 1st of June 2012 and harvested on the 23rd of November 2012.

Table 1 Physical and chemical properties of Condamine soils at 0-10 and 10-20 cm depths

Component	EC	pH	NH_4^+-N air dry	NO_3^--N air dry	Ca	Mg	Na	K	ESP	CEC	Cu	Zn	Mn	Fe
Units	dS m^{-1}	-	mg kg^{-1}	mg kg^{-1}	meq 100 g^{-1}	meq 100 g^{-1}	meq 100 g^{-1}	meq 100 g^{-1}	%	meq 100 g^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Depth (cm)														
0-10	0.05	6.4	3	15	11.5	2.84	0.253	1.83	1.4	18	1	1.5	111	34.1
10-20	0.04	6.9	4	6	12.8	3.6	0.88	1.05	4.2	21	0.9	0.2	98.1	28.5

EC: electrical conductivity; ESP: exchangeable sodium percentage; CEC: cation-exchange capacity.

2.1.2 Tillage treatments and sample collection

The field trial was arranged in a randomised complete block with four replications for each treatment, and the size of each plot was 12 × 100 m. One- or two-time chisel tillage (37.5 cm between tines) was applied on the 6th of March and 18th of April 2012 to a depth of 15 cm. Soil sampling was done on the 10th of April 2013. Seven soil samples were collected per plot along an imaginary Z shape. Experimental and sampling design of this field trial is shown in Fig. 1. The detailed history of the experimental field can be referred to Crawford et al. (2015) and related site information is shown in Fig. 2. Soil samples from the same depth of the same plot were composited in one bag on-site and were then transported to the laboratory where they were sieved (porosity < 4 mm) and tested for gravimetric water content immediately. Soil samples were stored at 4°C for subsequent tests.

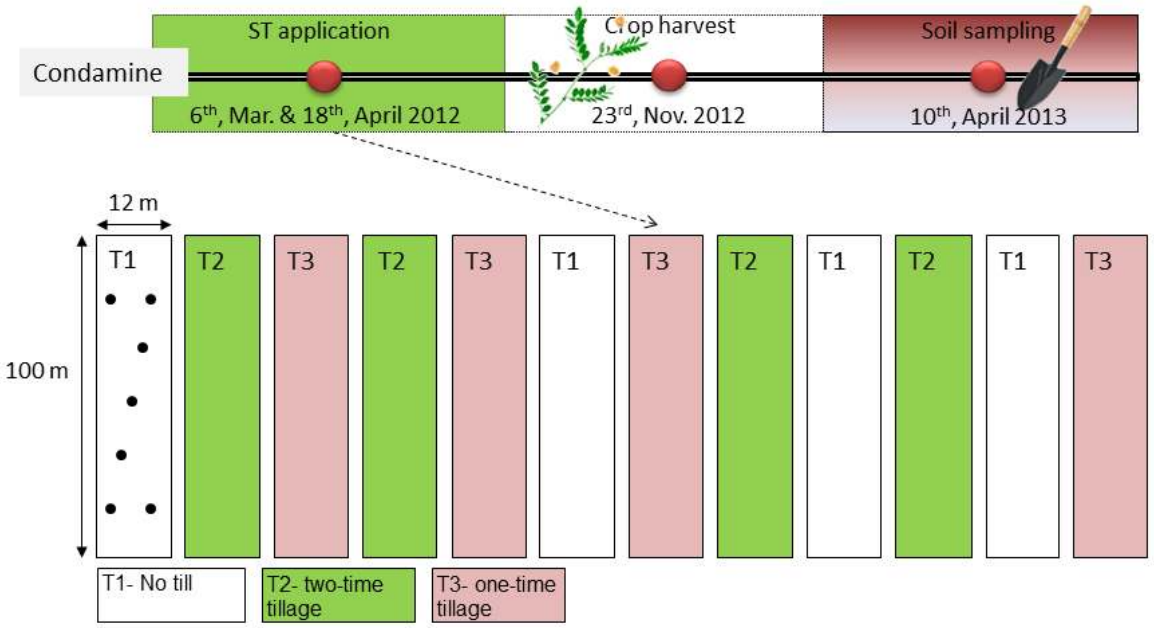

Fig. 1 Field experimental and sampling design for strategic tillage treatments with one-time and two-time chisel. A 9 meters' buffer space between plots was applied. Four plots per treatment are shown. The black dots shown in the figure represent the seven sampling points along a Z shape at each spot.

Fig. 2 Experiment site. **(a)** Site location in Australia; **(b)** Weed condition in NT soil during the fallow period of 2012; **(c)** Chisel implement (37.5 cm between tines) used in one- and two-time tillage; **(d)** Chisel-tilled soils during the fallow period of 2012; **(e)** In-crop weed condition in NT soil three months after tillage; **(f)** In-crop weed condition in chisel-tilled soil three months after

tillage. Weed populations in the NT was significantly suppressed three months after the implementation of one- or two-time tillage in 2012 (Crawford et al. 2015). Pictures b, c, d were taken on the date of tillage implementation, and e, f were taken three months after tillage.

2.2 Measurement of microbial biomass C and N (MBC/MBN)

Soil MBC and MBN levels were determined using the fumigation extraction method (Brookes et al. 1985). To start, 10 g of oven-dry equivalent fresh soil was fumigated with ethanol-free chloroform in a sealed glass desiccator for 72 h in the dark at room temperature. Soluble C from fumigated and non-fumigated soils was extracted with 50 mL 0.5 M K_2SO_4 at 250 rpm at room temperature for 1 h. The supernatant obtained by centrifugation (2,500 x g, 10 min) was subsequently filtered through No 1 filter paper (Watman). The total dissolved C was determined by a total C measurement instrument (Shimadzu TOC-VCSH). Both MBC and MBN were calculated as the difference between organic C or N of fumigated soils and organic C or N of non-fumigated soils divided by a constant soil-specific coefficient k_{EC} which is 0.45 for MBC and 0.54 for MBN (Brookes et al. 1985; Eiland and Nielsen 1996).

2.3 Patterns of C substrate utilisation

The physiological profiles were determined by the multi-Substrate Induced Respiration (SIR) approach using the MicroResp^{TM} system (James Hutton Institute, Invergowrie, Scotland, UK) (Campbell et al. 2003). A total of 15 pre-dispensed C sources were used as C substrates, which included carboxylic acids (citric acid, L-malic acid, methyl pyruvate, oxalic acid, D+galacturonic acid), amino acids (L-alanine, γ-aminobutyric acid, L-arginine), carbohydrates (β-d-fructose, D-glucose, D-(+)- trehalose, D- xylose, D+ cellubiose, mannitol) and one polymer (Tween 80) with milli-Q water used as a negative control. To prepare soil samples, 0.48 g soil was placed at the bottom of a deep-well plate. The moisture of all soil samples was modified to 30% with milli-Q water. Soil samples were incubated at 25°C for 3 days in a sealed plastic box containing a dish of self-indicating soda lime. Pre-dispensed C sources were added into each deep well according to Campbell et al. (2003) and three technical replicates were used for each C substrate. The detection microplate which was connected to a deep well plate contained 1% agar, 150 mM potassium chloride (KCl), 2.5 mM sodium bicarbonate ($NaHCO_3$) and 12.5 μg mL^{-1} cresol red. The assembled MicroResp^{TM} system was then incubated at 25°C for 6 h. Colour development on the detection microplate was measured at 570 nm after 6 h of incubation. The CO_2 production rate (μg CO_2-C g^{-1}

h^{-1}) was subsequently calculated from the difference between the absorbance at 6 h and the 0 h reading before exposure.

2.4 Total microbial activity (TMA)

The fluorescein diacetate (FDA) assay was performed according to Green et al. (2006). An aliquot of 15 mL potassium phosphate buffer (60 mM, pH 7.6) was added to two grams of soil in a falcon tube. To start the reaction, a volume of 200 µL of a 2 mg mL^{-1} FDA solution was added as substrate and shaken at 150 rpm, 30°C for 1 h. A volume of 950 µL from each reaction was taken and mixed with the same volume of 2:1 (v:v) chloroform/methanol thoroughly to stop the reaction. Three replicates were used in this step. The obtained mixture was subsequently centrifuged at 12,000 x g for 3 min, and 250 µL of the supernatant was aliquoted into 96 well plates and read at 450 nm in a microtiter plate reader (BMG Lab, Ortenberg, Germany).

2.5 Soil DNA extraction

Isolation of total DNA was performed by combining a soil sonication step prior to using the Powersoil DNA Isolation Kit (MOBIO Laboratories, CA) as previously described (Liu et al. 2016a). Two millilitres of the slurry obtained through sonication was centrifuged at 12,000 x g for 10 min and 0.25-0.50 g of the precipitated soils was used for the total DNA extraction using the Powersoil Kit. DNA concentration of each sample was determined through a Qubit™ fluorometer with Quant-iT dsDNA HS Assay Kits (Invitrogen).

2.6 Profiling of C and N cycling genes

The primers used and thermocycling conditions for qPCRs are displayed in Table 2. All qPCR reactions were carried out in a 10 µL system containing 5 µL master mix (Roche), 1.5 µL 0.3 µM primer pair, 1.0 µL DNA template (2.5 ng) and 2.5 µL nuclease free water in a LightCycler® 96. PCR conditions included 95°C for 900 s, 45 cycles of 95°C for 30 s, annealing for 45 s and 72°C (final elongation) for 60 s. The melting curve was obtained by using conditions 95°C for 10 s, 65°C for 60 s and 97°C for 1 s. qPCR results were analysed with data analysis software provided by Light Cycler® 96. Gradient temperatures (53-67°C) were used to optimise annealing temperatures using soil gDNA as templates for the six primers, and the optimised annealing temperatures are listed in

Table 2. Amplicons produced from soil gDNA using the six primer pairs were examined with agarose gel electrophoresis as shown in Supplementary Fig. 1. Bands of correct sizes on an agarose gel were excised and further purified with a commercial PCR cleaning Kit (Wizard® SV Gel and PCR Clean-Up System, Promega) and then sent to AGRF (Australian Genome Research Facility Ltd., Brisbane) for Sanger sequencing. By BLAST searches in NCBI, all amplification products were found to be correct and consistent with the function of the target gene amplified by each primer. No soil inhibition on qPCR reactions was observed by using 2.5 ng soil DNA per reaction (data not shown). For making a standard curve for each gene, purified PCR products were diluted to 2, 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, 2×10^6 and 2×10^7 copies per µL. Standard curves made for each gene were as follows: *Chitinase* A: y= -1.0196x+35.043 (R^2=0.9859), *amoA*: y= -1.2275x+33.518 (R^2=0.9999), *nifH*: y= -1.6161x+47.827 (R^2=0.9948), *narG*: y= -1.8342x+36.707 (R^2=0.9900), *nirK*: y= -1.0477x+25.289 (R^2=0.9925) and *nosZ*: y= -1.4453x+28.723 (R^2=0.9725). Gene abundance for a soil sample was obtained by comparing its Ct value against the corresponding standard curve.

Table 2 Primers and annealing temperatures used in the qPCR for profiling genes involved in carbon (C) and nitrogen (N) cycles.

Genes	Forward	Reverse	Amplicon size (bp)	Annealing temperature (°C)	Reference
nifH	AAAGGYGGWAT CGGYAARTCCACCAC	TTGTTSGCSGCRTA CATSGCCATCAT	459	60	(Rösch et al. 2002)
amoA	GGGGTTTCTACTGGTGGT	CCCCTCKGSAAAG CCTTCTTC	491	56	(Rotthauwe et al. 1997)
nirK	TCATGGTGCTGCCGCGK GACGGA	GAACTTGCCGGTK GCCCAGAC	326	63	(Yan et al. 2003)
nosZ	CGYTGTTCMTCGACAGC CAG	CATGTGCAGNGCR TGGCAGAA	700	64	(Rösch et al. 2002)
narG	TAYGTSGGSCARGARAA	TTYTCRTACCABG TBGC	650	59	(Philippot et al. 2002)
Chitinase A	CGTCGACATCGACTGGG ARTDBCC	ACGCCGGTCCAGC CNCKNCCRTA	400	63	(Yergeau et al. 2007)

2.7 Genetic profiling of the soil microbial communities

2.7.1 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Eubacterial 16S rRNA genes were amplified by PCR using 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3') as previously described (Liu

et al. 2016a). The forward primer was labelled with the fluorophore 6-FAM at the 5' end. Two amplification products were combined per sample and an aliquot of 20 µL was digested at 37°C for 2 h using 1 U of *Msp*I. Digested PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and then concentration was adjusted to 50 ng μL^{-1}. Samples were sent to AGRF (Melbourne) for fragment analysis using capillary electrophoresis (AB3730 DNA analyser, Applied Biosystems, Foster City, CA, USA).

2.7.2 16S rRNA Illumina MiSeq sequencing

Universal 16S rRNA genes were amplified by PCR using 926F and 1392R primers modified at the 5' end to contain the Illumina linker sequences 1 and 2, respectively. PCR conditions were as described by Liu et al. (2016a). Amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Inc.) and subjected to dual indexing using the Nextera XT Index Kit (Illumina) as per the manufacturer's instructions. Indexed amplicons were also purified using Agencourt AMPure XP beads and then quantified using a PicoGreen dsDNA Quantification Kit (Invitrogen). Equal concentrations of each sample were pooled and sequenced on an Illumina MiSeq at the University of Queensland's Institute for Molecular Biosciences (UQ, IMB) using 25% PhiX Control v3 (Illumina) and a MiSeq Reagent Kit v3 (600 cycle; Illumina) according to the manufacturer's instructions.

Primer sequences were removed from each fastq file using the QIIME v1.9.1 script multiple_extract_barcodes.py. The header line of each sequence was then modified to contain a sample ID using a custom bash script and each file was quality filtered using the QIIME script multiple_split_libraries.py with the homopolymer filter deactivated (Caporaso et al. 2010). The forward reads from each sample were concatenated into a single file and checked for chimeras against the October 2013 release of the GreenGenes database using UCHIME ver. 3.0.617 (Edgar et al. 2011). Homopolymer errors were corrected using Acacia (Bragg et al. 2012). Sequences were then subjected to the following procedures using QIIME: 1) sequences were clustered at 97% similarity using UCLUST, 2) GreenGenes taxonomy was assigned to the cluster representatives using BLAST, and 3) tables with the abundance of different Operational taxonomic unit (OTUs) and their taxonomic assignments in each sample were generated. The number of reads was rarefied to 2,400 sequences per sample by re-sampling the OTU table. Rarefaction curves were created for all 24 soil samples as shown in Fig. 5. The mean number of observed (Sobs) and predicted (Chao1) OTUs and Simpson's diversity index values were calculated using QIIME. The raw sequence read

data for soil samples have been deposited in the NCBI Short Read Archive (SAR) with accession number SRP076647.

2.8 Statistical analysis

Tillage and depth effects on soil MBC and MBN, TMA, utilisation of each C substrate, soil N and C cycling genes and soil microbial diversities were analysed by one-way analysis of variance (ANOVA) with post hoc comparison of means using Tukey's HSD at 95% confidence. For depth comparison of these parameters, two-tailed Student's t-test was performed. The one-way ANOVA was performed using the general ANOVA module in SPSS (IBM SPSS Statistics 23). Tillage and depth effects on utilisation of C substrates as well as composition of soil microbial communities (using T-RFLP and 16S rRNA deep sequencing) were investigated by PERMANOVA using R (Version 3.0.2). Potential tillage effect on specific OTUs was evaluated using two-tailed Student's t-test. Differences in the composition of microbial communities and the utilisation of C substrates between samples were visualised using Principal Component Analysis (PCA) and heatmaps.

3 Results

There were no significant ST effects on soil MBC or MBN at either soil depth irrespective of the chisel frequency used ($P > 0.05$, one way ANOVA) (Table 3). On average, MBC and MBN were all greater in soils sampled from 0-10 cm than in those taken from 10-20 cm ($P < 0.001$, ANOVA) (Table 3).

Table 3 Soil Microbial Biomass C (MBC, mg C g dry soil^{-1}), Soil Microbial Biomass N (MBN, mg N g dry soil^{-1}) and Total Microbial Activity (TMA, fluorescein μg mL^{-1} g^{-1} soil h^{-1}) as indicated by FDA hydrolysis between treatments and the NT. Distinct lowercase letters indicate significant differences between treatments and distinct uppercase letters indicate significant differences between depths. Errors represent standard deviations of each mean (n=4).

	Depth	NT	one-time	two-time
MBC	0-10 cm	0.49±0.11^{aA}	0.63±0.09^{aA}	0.63±0.06^{aA}
	10-20 cm	0.13±0.03^{aB}	0.12±0.01^{aB}	0.09±0.01^{aB}
MBN	0-10 cm	0.015±0.006^{aA}	0.019±0.005^{aA}	0.019±0.010^{aA}
	10-20 cm	0.007±0.002^{aB}	0.008±0.002^{aB}	0.008±0.002^{aB}
TMA	0-10 cm	2.31±0.19^{aA}	2.36±0.33^{aA}	2.57±0.56^{aA}
	10-20 cm	0.85±0.14^{aB}	0.97±0.35^{aB}	1.52±0.47^{aA}

Soil CLPP was analysed by PCA as shown in Fig. 3a. The PC1 and PC2 axis explained 55.1% and 18.7% of the total variance, respectively. Overall, C substrate utilisation patterns were not influenced by ST, irrespective of the tillage frequency (Fig. 3a, $P = 0.654$, PERMANOVA). Two-time tillage increased the average C substrate utilisation (+62.47%) based on fifteen C sources

(Fig. 3b; $P = 0.032$). No significant differences between ST treatments and the NT were observed at either 0-10 cm or 10-20 cm soil depth when each C substrate was analysed independently. However, the utilisation of L-malic acid ($P = 0.09$), β-d-fructose ($P = 0.10$) and D-glucose ($P = 0.06$) were marginally higher than that of the NT at 10-20 cm soil depth after two-time chisel tillage (Fig. 3b). Depth effects on the utilisation of C substrates were not significant except that oxalic acid was greater utilised in 10-20 cm soil depth than in 0-10 cm soil depth (Fig. 3b).

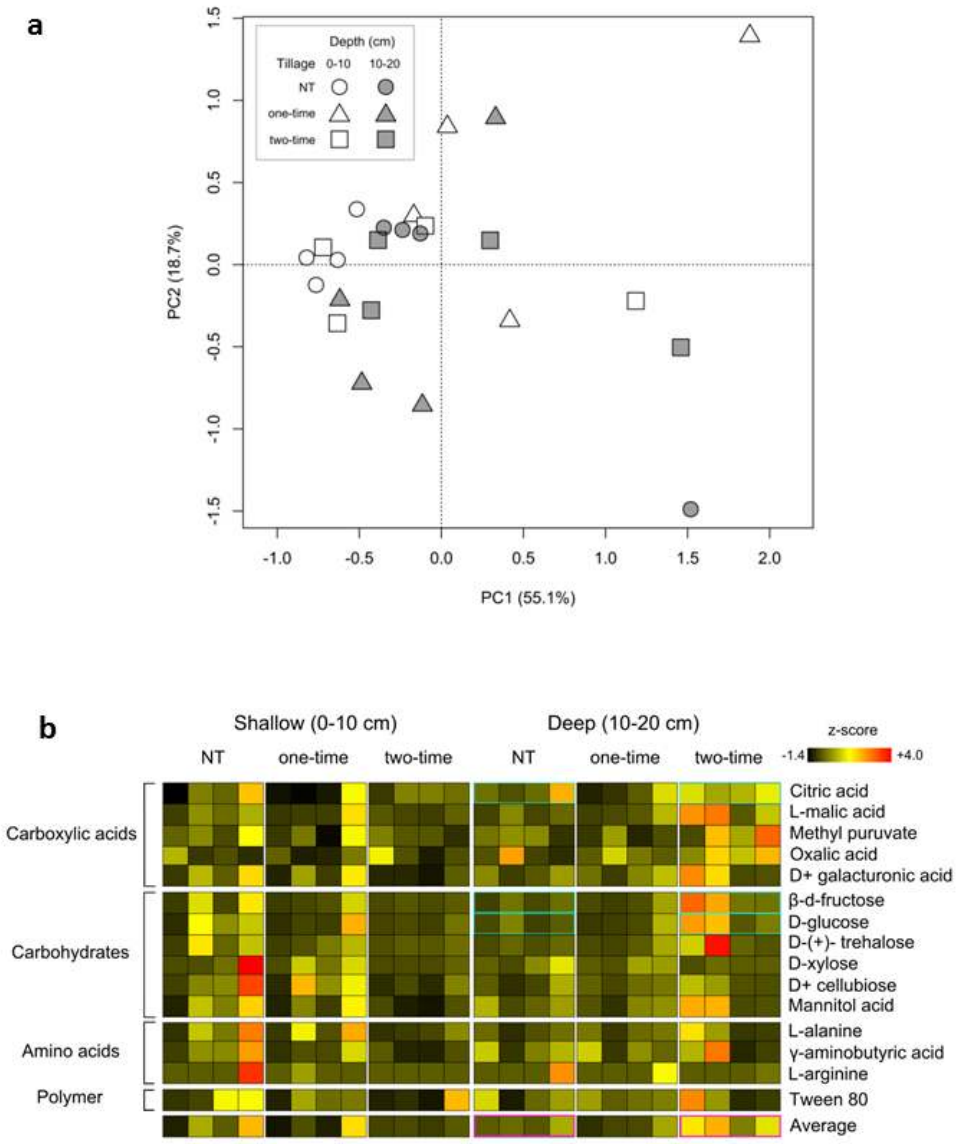

Fig. 3 Principal Component Analysis (PCA) ordination **(a)** and heatmap **(b)** summarising variation in the C substrate utilisation profiles between samples based on the z-score transformed C-utilisation (CO_2 evolution) data. The green boxes indicate that the utilisations of β-d-fructose, D-glucose and L-malic acid were marginally significantly greater in the chisel-tilled soils at 10-20 cm depth when compared with that of the NT ($P < 0.1$). The pink box showed a significantly higher

average C substrate utilisation rate in two-time chisel-tilled soils at 10-20 cm than that of the NT ($P = 0.032$).

Soil TMA, as indicated by the rates of FDA hydrolysis, was not influenced by ST ($P > 0.05$) (Table 3). Although ST effects on soil TMA were not significant, the mean value was somewhat higher in the two-time chisel tilled soils than that of the NT (Table 3). Differing from the NT and one-time chisel treatment, depth effect on FDA hydrolysis was absent in the two-time chisel-tilled soils.

To assess ST effects on C and N cycling potentials of the NT soils, gene abundances of bacterial *Chitinase* (group A), *nifH*, *amoA*, *narG*, *nirK* and *nosZ* were determined using qPCR. A single band of the correct size, visualised on a 1.5% agarose gel using these six primer pairs, confirmed the specificities of qPCR amplifications (Supplementary Fig. 1). Overall, ST did not cause any significant changes in the abundance of these genes at either 0-10 cm or 10-20 cm soil depth ($P > 0.05$) (Table 4). Two-time tillage may have led to a slight change in the distribution of *Chitinase A*-producing bacteria as higher abundance of this gene in 10-20 cm soil depth than in 0-10 cm soil depth was observed, which distinguished two-time tillage from the NT and one-time tillage. Depth effects on gene abundances of *nifH*, *amoA* and *narG* were evident ($P < 0.001$) but not for *nirK* or *nosZ* (Table 4).

Table 4 Abundance of bacterial functional genes *Chitinase A*, *nifH*, *amoA*, *nosZ*, *narG* and *nirK* in no till, one-time and two-time strategic tillage at soil depths of 0-10 cm and 10-20 cm. Distinct lowercase letters indicate significant differences between treatments and distinct uppercase letters indicate significant differences between depths. Standard deviations are shown beside the means (n=4).

	Depth	NT	one-time	two-time
chitinase	0-10 cm	1434.67±297.05^{aA}	1494.46±256.48^{aA}	1104.27±73.13^{aA}
A	10-20 cm	2291.48±873.97^{aA}	1546.86±188.01^{aA}	1410.72±90.82^{aB}
nifH	0-10 cm	1066.21±159.43^{aA}	1379.50±556.20^{aA}	1512.30±585.77^{aA}
	10-20 cm	519.00±129.50^{aB}	370.63±42.87^{aB}	748.54±208.75^{aA}
amoA	0-10 cm	220.08±8.63^{aA}	209.78±25.97^{aA}	216.78±27.04^{aA}
	10-20 cm	139.51±15.85^{aB}	170.03±37.42^{aA}	149.42±9.32^{aB}
narG	0-10 cm	7.62±1.07^{aA}	6.84±1.01^{aA}	8.0±1.00^{aA}
	10-20 cm	4.71±0.71^{aB}	4.90±0.83^{aA}	4.89±0.79^{aB}
nirK	0-10 cm	14.53±1.45^{aA}	13.98±1.43^{aA}	14.43±1.38^{aA}
	10-20 cm	12.04±1.33^{aA}	15.99±1.47^{aA}	13.48±11.40^{aA}
nosZ	0-10 cm	6.88±0.98^{aA}	6.95±0.78^{aA}	7.73±1.16^{aA}
	10-20 cm	5.89±0.73^{aA}	5.46±0.73^{aA}	6.60±1.07^{aA}

Only those restriction fragments (T-RFs) with sizes within 65-500 bp were considered in the data analysis (Fig. 4). A total of 105 effective T-RFs were obtained for the Solonetz soil. ST did not change the composition of soil bacterial communities at either 0-10 cm or 10-20 cm soil depth when using T-RFLP for assessing this difference. However, the depth effect was evident ($P < 0.001$, PERMANOVA) (Fig. 4a,b). PCA revealed that PC1 and PC2 explain 55.2% and 9.58% of the total variation between treatments along the first and the second axis, respectively (Fig. 4a).

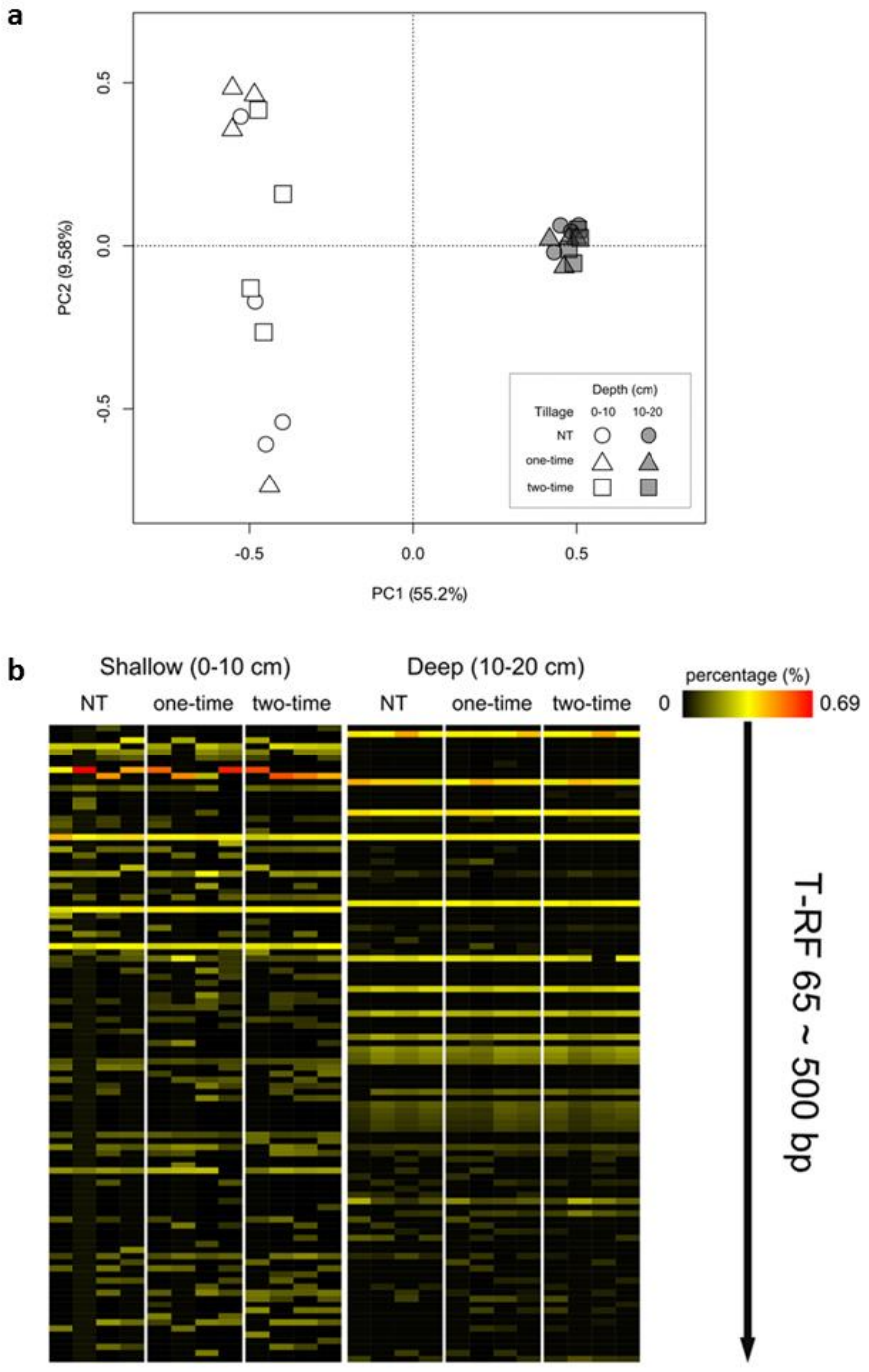

Fig. 4 Principal Component Analysis (PCA) ordination summarising variation in the composition of bacterial communities between samples using the T-RFLP method **(a)**. Heatmap depicting the frequencies of T-RFs detected in different soil samples **(b)**. Colour change from black to red indicates the percentages of each T-RFs changing from 0% to 0.69%. Only T-RFs of length between 65 to 500 bp were included in the analysis.

ST effects on microbial communities using 16S rRNA sequencing are shown in Fig. 6. PCA revealed that the PC1 and PC2 explained 44.2% and 21.7% of the total variance, respectively. Changes in the composition of microbial communities in response to ST at both depths were not detected, irrespective of the frequency used ($P = 0.35$, 0-10 cm; $P = 0.73$, 10-20 cm, PERMANOVA) (Fig. 6a). Furthermore, the mean number of observed (Sobs) and predicted (Chao1) OTUs and Simpson's diversity index values were not influenced by ST at both soil depths regardless of the tillage frequency used (Table 5, $P > 0.05$). Rarefaction curve analysis also did not reveal significant differences between treatments at either soil depth (Fig. 5). The composition of microbial communities differed between depths ($P < 0.001$, PERMANOVA) (Fig. 6a, b). An enrichment of Acidobacteria RB41 [4] (+341%) and Acidobacteria iii1-15 [8] (+248%) at 10-20 cm soil depth in one-time chisel-tilled soils relative to the NT soils was observed (Fig. 6b). Another two abundant bacterial OTUs (> 1.0%) which were also affiliated to Acidobacteria (RB41 [1] and iii-15 [26]) were marginally significantly enriched by one-time chisel tillage relative to the NT at 10-20 cm depth ($P = 0.057$ and $P = 0.080$, respectively) (Fig. 6b). Microbial communities at 0-10 cm depth were associated with larger relative abundances of Acidobacteria RB41 ([1] and [4]) and Acidobacteria iii1-15 ([8] and [26]), while those at 10-20 cm depth were associated with larger relative abundances of Actinobacteria, including Nocardiodaceae (family) [2], *Solirubrobacter* (genus) [6], Micrococcaceae (family) [7], *Arthrobacter* (genus) [15], *Kribbella* (genus) [16], Micrococcaales (order) [17], 0319-7L14 (order) [27], and *Promicromonospora* [28] (Fig. 6a, b). Interestingly, all abundant Actinobacteria OTUs were more abundant at 10-20 cm depth than 0-10 cm as shown in Fig. 6a.

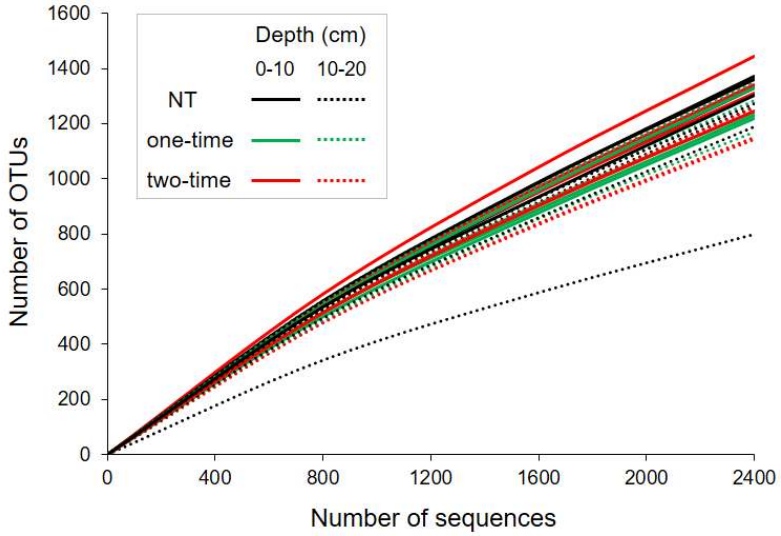

Fig. 5 Rarefaction curves of the microbial communities describing the discovering numbers of OTUs (Y) against the number of sequences sampled (X). Four replicates per treatment are shown. OTUs = Operational Taxonomic Units.

Table 5 The mean number of observed (Sobs) and predicted (Chao1) OTUs and Simpson's diversity index values of bacterial communities associated with the NT, one-time tillage and two-time tillage. Distinct lowercase letters indicate significant differences between treatments and distinct uppercase letters indicate significant differences between depths. Standard deviations are shown beside the means (n=4). Values are rarefied means based on 25 reasamplings of 2400 individual sequences per sample.

	Depth	NT	one-time	two-time
Chao 1	0-10 cm	5347.9±83.6^{aA}	4950.5±145.8^{aA}	4928.4±267.4^{aA}
	10-20 cm	4400.9±627.4^{aA}	5290.0±278.9^{aA}	5028.8±327.6^{aA}
Observed OTU	0-10 cm	1341.6±21.2^{aA}	1256.0±30.9^{aA}	1331.7±47.0^{aA}
(richness)	10-20 cm	1132.6±127.6^{aA}	1293.7±47.4^{aA}	1229.8±56.7^{aA}
Simpson's	0-10 cm	0.9937±0.0009^{aA}	0.9931±0.0016^{aA}	0.9953±0.0013^{aA}
Diversity Index	10-20 cm	0.9832±0.0109^{aA}	0.9954±0.0010^{aA}	0.9906±0.0038^{aA}

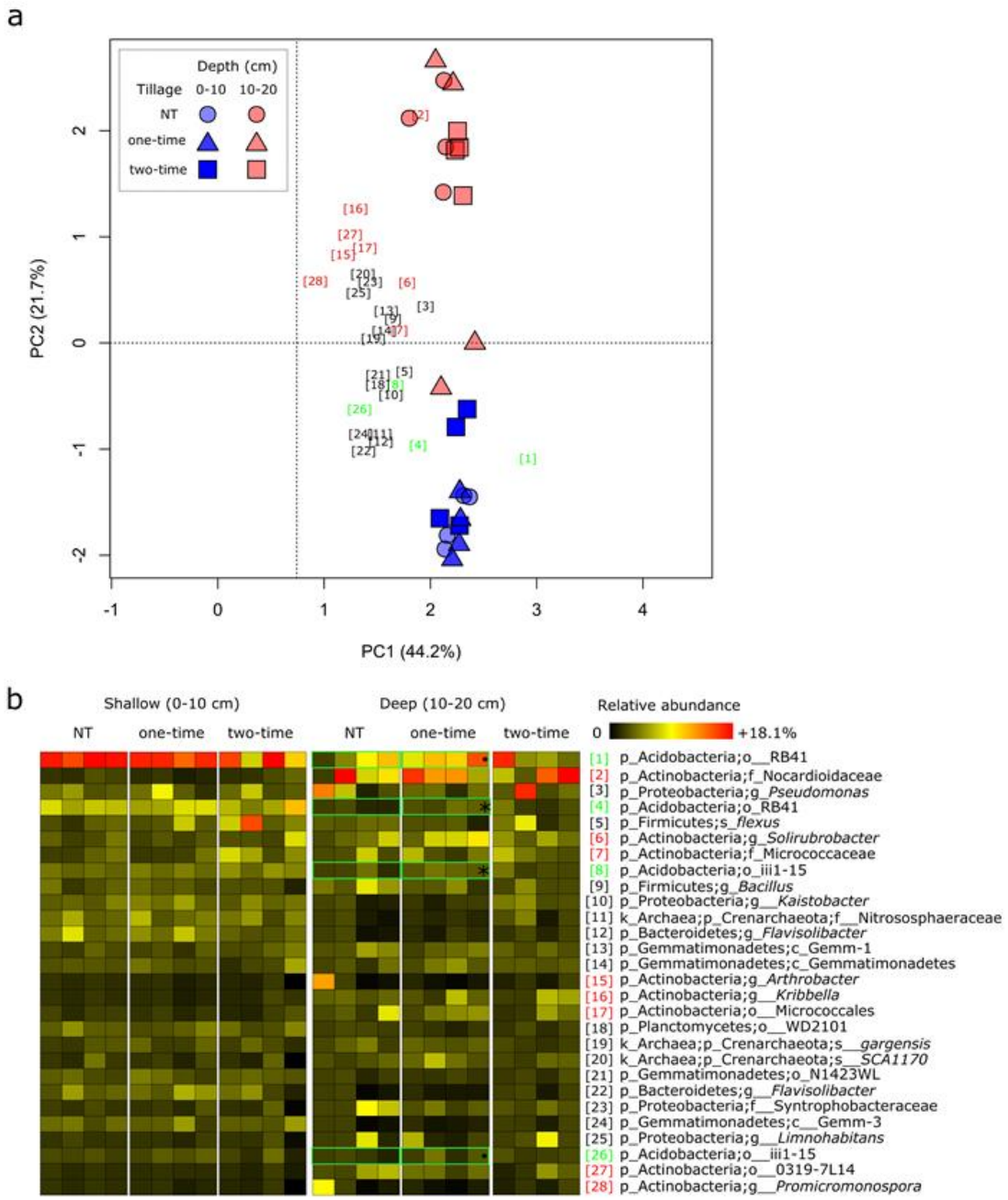

Fig. 6 Principal Component Analysis (PCA) ordination **(a)** and heatmap **(b)** summarising differences in the composition of microbial communities between samples as indicated by Illumina deep sequencing of full-length 16S rRNA gene amplicons. In **a**, bacterial OTUs ([2], [6], [7], [15], [16], [17], [27] and [28]) highlighted in red were affiliated to Actinobacteria while those bacterial OTUs ([1], [4], [8] and [26]) highlighted in green were affiliated to Acidobacteria. In 4**b**, those samples highlighted with blue squares were affiliated to Acidobacteria that were increased in relative abundances after one-time chisel tillage ($P < 0.1$ $^{(.)}$, $P < 0.05$ $^{(*)}$). Numbers [1] to [28] in **a** and **b** correspond to the same OTUs.

4 Discussion

In general, our results support the hypothesis that after the harvest of a chickpea cropping season, effects of ST applied with either one- or two-time chisel on soil microbial properties are small. Strategic tillage only caused a slight increase in relative abundance of Acidobacteria RB41 and Acidobacteria iii1-15 or utilisation of C substrates at 10-20 cm soil depth. All the other tested soil microbial parameters of the NT including MBC and MBN, TMA, abundance of some C and N cycling genes, and the composition of soil microbial communities were not influenced by ST.

4.1 ST effects on soil MBC and MBN

Soil microorganisms are the crucial component of soil ecosystem responsible for the decomposition of organic matter, nutrient cycling and energy flow (Schloter et al. 2003). It can therefore provide potentially vital information for determining the impact of tillage or any other soil structural changes. The application of chisel tillage did not result in changes in soil MBC or MBN on this long-term NT managed Solonetz. Consistently, Crawford et al. (2015) reported minimal changes in soil chemical and physical properties after tillage treatments. The levels of soil organic carbon (SOC) can hold the key to understanding possible changes that may occur due to tillage. Despite the fact that chisel tillage incorporates a certain amount of stubble and crop residues up to 10 cm as indicated by previous studies, SOC holds a steady level over a long timeframe (Logsdon 2013; Raper 2002). Due to an unchanged SOC (Crawford et al. 2015), only minor changes in MBC and MBN were expected here.

Microbial communities of long-term NT managed soils have the ability to resist disturbance and recover to the NT conditions within a certain timeframe, typically known as soil biological resistance and resilience (Allison and Martiny 2008; Kuan et al. 2007). For instance, soil bacteria and Archaea can revive from suppressed conditions quickly due to their fast growth and high degree of physiological flexibility as well as rapid evolution (Allison and Martiny 2008). A key parameter of ST was to avoid depletion of soil moisture and minimise the disruption of aggregates (Dang et al. 2015b). In the present study, the chisel implement and the timings of tillage application were used to minimise disturbance. The minimal soil inversion nature of chisel tillage and low frequency tested may have allowed the soil to return to pre-disturbance conditions within one-year post-ST (de Moraes Sá et al. 2014; Gregory et al. 2009). It is also plausible that the application of tillage simply did not create enough disturbances to reduce the abundance of some microbial populations and

hence produce a measureable impact. Recent studies showed that conventional tillage with a soil inversion implement of MP reduced soil MBC and MBN to different extents (López-Garrido et al. 2011; Melero et al. 2011; Wortmann et al. 2008, 2010). Therefore, using minimal soil inversion implements such as chisel can potentially conserve soil microbial biomass when performing ST in the NT soils.

The MBC determined in our study (0.25-0.83 mg C g^{-1} soil at 0-10 cm depth and 0.07-0.22 mg C g^{-1} soil at 10-20 cm depth) falls within the recorded range in Australian agricultural systems. Generally, MBC ranges from 1.0% to 5.0 % of total organic C (Gonzalez-Quiñones et al. 2011; Jenkinson and Ladd 1981), but the typical Australian soil only from 2.0% to 4.0% (Bell et al. 2006; Gonzalez-Quiñones et al. 2011). In our study, Cmic: Corg ranged from 5.6% to 7.3% and 1.7% to 2.4% at soil depths of 0-10 cm and 10-20 cm, respectively. Given that the percentage seldom exceeds 5.0% (Gonzalez-Quiñones et al. 2011), it could be interpreted that the Solonetz in this study may be more fertile than other reported Australian soils. The texture contrast observed (clay loam sandy over light medium clay) at this site possibly holds the answer to this result as previous research by Bell et al. (2006) was undertaken on uniform clays (Vertosols). Further investigation is required to assess the possibility that the presence of a clear or abrupt textural change is the reason for the higher percentage.

4.2 ST effects on soil microbial activity

4.2.1 ST effects on soil TMA

One- or two-time tillage did not influence TMA at either soil depth, but two-time tillage resulted in minimal stratification of TMA as the depth effect was absent (Table 3). It is likely that two-time chisel tillage could have mixed soil between depths while one-time tillage did not. FDA activity includes esterase, lipase and certain protease activities (Caldwell 2005). These enzyme activities have shown higher stability towards mechanical disturbance than others such as cellulase and laccase (Chaer et al. 2009). FDA analysis suggests that major microbial functions associated with decomposition may have been maintained in ST treatments. The FDA hydrolysis assay has been used for determining the impacts of management practices on soil biota in Vertosols in Northern Grains Region of Australia, where the TMA ranged from 1.2 to 5.4 µg mL^{-1} g^{-1} soil h^{-1} FDA for all soil samples tested (Bell et al. 2006). Our previous studies also used the FDA method for measuring TMA of Australian soils, which ranged from 0.52 to 1.52 µg mL^{-1} g^{-1} soil h^{-1} at 0-10 cm and from

0.27 to 0.73 µg mL^{-1} g^{-1} soil h^{-1} at 10-20 cm for a Calcisol soil, and from 0.6 to 0.8 µg mL^{-1} g^{-1} soil h^{-1} at 0-10 cm and from 0.3 to 0.5 µg mL^{-1} g^{-1} soil h^{-1} at 10-20 cm for a Vertisol soil (Liu et al. 2016a,b). The TMA of the Solonetz in the present study (2.31-2.57 µg mL^{-1} g^{-1} soil h^{-1}, 0-10 cm; 0.85-1.52 µg mL^{-1} g^{-1} soil h^{-1}, 10-20 cm) are higher than values reported for the other soil types. This provides another contrast with previous results on uniform clays, and warrants further investigation into texture contrast soils. These findings help to build a TMA database for Australian soils that has predominately uniform texture TMA values, expanding the information available for soil management decisions.

4.2.2 ST effects on soil CLPP

Microbial mineralisation and immobilisation of nutrients by soil microorganisms and enzyme activities strongly influence soil fertility (Schloter et al. 2003). MicroResp™ is a quick and effective method for assessing soil C substrate utilisation ability (Campbell et al. 2003). In this study, the average utilisation rate of 15 C sources was increased by two-time chisel tillage, but it did not influence any separate C substrate (Fig. 3b). The high variance among replicates of each treatment contributed to the non-significant differences in the comparison of results. Shannon diversity and evenness did not show significant differences between treatments and soil depth (data not shown). Soil physicochemical conditions could have bigger impacts on soil microbial diversity and soil function than agricultural management practices. For instance, pH is the key physicochemical parameter related to soil capacity to catabolise different C-substrates and soil biological diversity in Australian agricultural soils under different soil managements (Wakelin et al. 2008). Therefore, our results can be a reflection of the absence of changes in soil physicochemical properties due to ST as reported in our previous study (Crawford et al. 2015). The MicroResp™ assay supported the results obtained with the TMA assays. Despite higher values in MBC and TMA, the average C utilisation rates in the present study were not different from the previously reported two soils, Vertisol and Calcisol (Liu et al. 2016a,b).

4.3 ST effects on soil C and N cycling genes

Bacterial Chitinase (group A), encoded by *Chitinase* gene, is produced in abundance by a variety of bacteria, such as Streptomycetes (Hamid et al. 2013). Chitinases degrade chitin in the environment to supply bacteria with C and N sources as well as energy (Hamid et al. 2013). Strategic tillage did not influence the abundance of *Chitinase A* at either soil depth, but two-time chisel tillage led to a

depth effect, which was absent in one-time tillage and the NT. It is possible that a slight redistribution of *Chitinase A*-producing organisms between depths occurred. In the two-time tillage treatment, no differences in abundance of members of Actinobacteria, which typically have chitinolytic activity (e.g. *Streptomyces* sp.) were detected between depths (Nagpure et al. 2014). However, various fungi are also able to produce chitinases and they have not been profiled in the present study, and therefore may have caused this difference in *chitinase A* abundance between depths.

Biological N fixation refers to the metabolic process that converts atmospheric N_2 into biologically available forms by diatrophs and this process is of paramount importance for terrestrial ecosystems (Dixon and Kahn 2004). This process is catalysed by nitrogenase. A subunit of this enzyme is encoded by the *nifH* gene, which can be used as a marker to study the distribution of nitrogen-fixing microbes (also known as diazotrophs) in the environment without the need for cultivation. The *nifH* gene is present in the genome of free-living nitrogen-fixing bacteria and in symbiotic bacteria (e.g. *Rhizobium* spp.) associated with a wide range of plants. This useful biomarker has been widely used to investigate the effects of soil management on nitrogen-fixing bacteria (Hayden et al. 2010). Nitrification is the biological oxidation of ammonia (NH_3) to nitrate (NO_3^-) which is carried out by specific groups of ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB) (Li et al. 2011) using the ammonia monooxygenase encoded by the *amoA* gene (Rotthauwe et al. 1997). Strategic tillage did not significantly affect *nifH* or *amoA* gene abundances (Table 4), which indicates that ST possibly maintained similar levels of ecosystem functions associated with N fixation and nitrification in NT soils.

Denitrification is the stepwise reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen gas (N_2), which is carried out by a phylogenetically diverse group of anaerobic bacteria (Knowles 1982). The reduction reaction of nitrite (NO_2^-) to nitric oxide (N_2O) is catalysed by either a cytochrome cd1 enzyme encoded by *nirS* or a Cu-containing enzyme encoded by *nirK* driven by nitrate-respiring bacteria. In the last step of the denitrification pathway, nitrous oxide (N_2O) is reduced to N_2 by a N_2O reductase encoded by the *nosZ* gene in bacteria and archaea. In our research, the quantification of the abundance of the genes involved in nitrate (NO_3^-) (*nirK*) and nitrous oxide (N_2O) (*nosZ*) reduction showed no general trends associated with ST. Nitrogen losses from agriculture systems account for most of the emissions of N_2O which contributes to global warming and ozone depletion in the stratosphere (Yu et al. 2014). Similar results to our study have been reported previously as one-time summer tillage during dry periods did not alter the emission magnitude of N_2O in a long-term NT (Norton et al. 2014). Most bacteria harbouring *nosZ* belong to

a wide range of various subclasses of Proteobacteria and some Archaea. No measured changes in abundance of either α, β and γ Proteobacteria or Archaea (Fig. 6b) were consistent with the unchanged gene abundance of *nosZ* (Table 4).

However, measurement of abundance of soil N and C cycling genes using DNA-based qPCR method does not necessarily correspond to the functions taking place at the moment of soil sampling. Our study provides information about potential changes in C and N after ST. It is worth pointing out that detecting functional genes on DNA samples does not mean that enzymes which they encode have been synthesised. Moreover, functional gene quantification using cDNA derived from RNA is more powerful than using genomic DNA as a template to study ongoing soil functions given that transcript abundances are measured. Other methods including stable isotope probing provide a more direct assessment of the dynamics of C and N transformations in soil (Li and Lang, 2014).

4.4 ST effects on the composition of soil microbial communities

From our results obtained with T-RFLP and 16S rRNA deep sequencing, we concluded that ST with chisel led to only small changes in the overall composition of soil microbial communities of the NT soils. Larger relative abundances of Acidobacteria and Actinobacteria were observed respectively in 0-10 cm and 10-20 cm soil depths of this Solonetz (Fig. 6). Fierer et al. (2003) found that gram-positive bacteria such as Actinobacteria easily gained an increased proportion of the total microbial communities in progressively deep soil while gram-negative bacteria (e.g. Acidobacteria), fungi and protozoa inclined to have a higher abundance in shallow soils, which are richer in nutrients. Interestingly, we found that Acidobacteria RB41 (2.75% in proportion) and Acidobacteria iii1-15 (0.66%), the two most abundant Acidobacteria OTUs in this Solonetz soil, increased in relative abundance at 10-20 cm soil depth after one-time tillage (Fig. 6). It could be inferred from the soil biochemical properties that this Solonetz soil is slightly acidic at the surface (pH = 6.4) while neutral at deep layers (pH = 6.9) (Table 1). Acidobacteria are acidophilic, and the higher abundance of these bacteria at the surface compared to deeper soil layers is consistent with the pH of this soil (Sait et al. 2006). However, the increase in proportion of Acidobacteria only occurred after a one-time tillage event but not two-time tillage, and the exact reason for this warrants further study. Members of the Acidobacteria display similar functional capabilities as Bacteroidetes and specialise on degradation of plant-derived organic matter (Naumoff and Dedysh 2012). Whether this slight increase of soil Acidobacteria impacts on soil health and plant performance still warrants further studies.

It is possible that soil microbial communities had recovered to the NT conditions 1 year after ST. Our previous studies also did not find major ST effects on the composition of soil microbial communities of different soil types (Calcisol and Vertisol) in short-term in Northern Grains Region (NGR) of Australia (Liu et al. 2016a,b). Basically, the resilience and/or resistance of long-term NT managed soils may have led to the absence of changes in the composition of soil microbial communities as a consequence of ST. There have been other consistent studies on the influence of one-time tillage on the composition of soil microbial communities, e.g. Rincon-Florez et al. (2016) reported that the composition of microbial communities was not altered by ST using less inversion implements with chisel plow sweeps and offset disc in long-term NT Vertosols 17 weeks after tillage application.

Similarly to the present study, Actinobacteria have also been the dominant bacterial groups in soil types of Leptic Regosols, Eutric cambisol, Vertisol and Calcisol worldwide as measured by qPCR (Liu et al. 2016a,b; Philippot et al. 2011; Wessén et al. 2010). A higher proportion of Actinobacteria in surface soil compared to deeper soils was also found in an Australian Calcisol soil by 16S taxonomic method using qPCR (Liu et al. 2016b). Solonetz is a texture contrast soil with clay loam sandy at 0-10 cm and light medium clay at 10-20 cm, and this may have contributed to the difference in bacterial communities between depths.

5 Conclusions

In this study, the changes of microbial properties one year after ST during the summer fallow period were investigated using multiple approaches. An increase in relative abundance of Acidobacteria and a trend of an increase in soil microbial activity was observed after ST. Overall, ST with chisel implement did not cause major impacts on the soil biological parameters tested. The higher number of Acidobacteria in 0-10 cm relative to 10-20 cm soil depth defined the characteristics of this slightly acidic Solonetz, which could be used for reference in future research on similar soil types. We provide comprehensive data on DNA-based abundance of genes related to key steps of the N and C cycles along with the microbial communities of this acidic Solonetz. No consistent evidence for deleterious effects of ST on soil N and C cyclings could be found. In conclusion, ST had no detrimental short-term impact on soil microbiological indicators of soil health measured in this study. ST may be a suitable strategy to address issues faced by growers in NT systems without negating NT soil benefits; however, it is not known yet if the ST with chisel influences several other

soil parameters such as soil infiltration and water holding capacity, run-off of soils and nutrients, which may warrant more future experiments in-depth. Additionally, further long-term studies are also needed in this texture contrast soil to identify the possible higher TMA and higher MBC/MBN. The combination of a diverse range of classical and molecular techniques used in this study provided a useful toolbox to measure the impact of disturbances on soil microbial communities.

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Conflict of interest

The authors declare no conflict of interest.

References

Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci USA 105:11512-11519 doi:10.1073/pnas.0801925105

Bayer C, Martin-Neto L, Mielniczuk J, Pavinato A, Dieckow J (2006) Carbon sequestration in two Brazilian Cerrado soils under no-till. Soil Till Res 86:237-245 doi:http://dx.doi.org/10.1016/j.still.2005.02.023

Bell M, Seymour N, Stirling GR, Stirling AM, Van Zwieten L, Vancov T, Sutton G, Moody P (2006) Impacts of management on soil biota in Vertosols supporting the broadacre grains industry in northern Australia. Soil Res 44:433-451 http://dx.doi.org/10.1071/SR05137

Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW (2012) Fast, accurate error-correction of amplicon pyrosequences using Acacia. Nat Meth 9:425-426 doi:10.1038/nmeth.1990

Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil nitrogen: A rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol Biochem 17:837-842 doi:http://dx.doi.org/10.1016/0038-0717(85)90144-0

Caldwell BA (2005) Enzyme activities as a component of soil biodiversity: a review. Pedobiologia 49:637-644 doi:10.1016/j.pedobi.2005.06.003

Campbell C, Janzen H, Juma N (1997) Case studies of soil quality in the Canadian Prairies: Long-term field experiments. Dev Soil Sci 25:351-398 doi:10.1016/S0166-2481(97)80044-X

Campbell CD, Chapman SJ, Cameron CM, Davidson MS, Potts JM (2003) A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl Environ Microbiol 69:3593-3599 doi: 10.1128/AEM.69.6.3593-3599.2003

Caporaso JG et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7:335-336 doi:10.1038/nmeth.f.303

Chaer G, Fernandes M, Myrold D, Bottomley P (2009) Comparative resistance and resilience of soil microbial communities and enzyme activities in adjacent native forest and agricultural soils. Microbial Ecol 58:414-424 doi: 10.1007/s00248-009-9508-x

Crawford M, Rincon-Florez V, Balzer A, Dang Y, Carvalhais L, Liu H, Schenk P (2015) Changes in the soil quality attributes of continuous no-till farming systems following a strategic tillage. Soil Res 53:263-273 http://dx.doi.org/10.1071/SR14216

Dang YP, Seymour NP, Walker SR, Bell MJ, Freebairn DM (2015a). Strategic tillage in no-till farming systems in Australia's northern grains-growing regions: I. Drivers and implementation. Soil Till Res 152, 104-114. doi:http://dx.doi.org/10.1016/j.still.2015.03.009

Dang Y, Moody P, Bell M, Seymour N, Dalal R, Freebairn D, Walker S (2015b). Strategic tillage in no-till farming systems in Australia's northern grains-growing regions: II. Implications for agronomy, soil and environment. Soil Till Res 152, 115-123 doi:10.1016/j.still.2014.12.013

de Moraes Sá JC, Tivet F, Lal R, Briedis C, Hartman DC, dos Santos JZ, dos Santos JB (2014) Long-term tillage systems impacts on soil C dynamics, soil resilience and agronomic productivity of a Brazilian Oxisol. Soil Till Res 136:38-50 doi:http://dx.doi.org/10.1016/j.still.2013.09.010

Derpsch R, Friedrich T, Kassam A, Li H (2010) Current status of adoption of no-till farming in the world and some of its main benefits. Int J Agric Biol Eng 3:1-25 doi: 10.3965/j.issn.1934-6344.2010.01.0-0

Dixon R, Kahn D (2004) Genetic regulation of biological nitrogen fixation. Nat Rev Micro 2:621-631 doi:10.1038/nrmicro954

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-2200 doi: 10.1093/bioinformatics/btr381

Eiland F, Nielsen JrD (1996) A method using peroxydisulphate digestion for total N analyses in soils and in soil microbial biomass extracts. Acta Agric Scand Sect B - Soil & Plant Science 46:81-85 doi:10.1080/09064719609413119

Engel F, Bertol I, Ritter S, Gonzalez AP, Paz-Ferreiro J, Vazquez EV (2009) Soil erosion under simulated rainfall in relation to phenological stages of soybeans and tillage methods in Lages, SC, Brazil. Soil Till Res 103:216-221 doi:10.1016/j.still.2008.05.017

Fierer N, Schimel JP, Holden PA (2003) Variations in microbial community composition through two soil depth profiles. Soil Biol Biochem 35:167-176 doi:http://dx.doi.org/10.1016/S0038-0717(02)00251-1

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA. (2004) Nitrogen cycles: past, present, and future. Biogeochemistry 70:153-226 doi:10.1007/s10533-004-0370-0

Gonzalez-Quiñones V, Stockdale EA, Banning NC, Hoyle FC, Sawada Y, Wherrett AD,Jones DL, Murphy DV. (2011) Soil microbial biomass—Interpretation and consideration for soil monitoring. Soil Res 49:287-304 http://dx.doi.org/10.1071/SR10203

Green V, Stott D, Diack M (2006) Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. Soil Biol Biochem 38:693-701 doi:10.1016/j.soilbio.2005.06.020

Gregory AS, Watts CW, Griffiths BS, Hallett PD, Kuan HL, Whitmore AP (2009) The effect of long-term soil management on the physical and biological resilience of a range of arable and grassland soils in England. Geoderma 153:172-185 doi:http://dx.doi.org/10.1016/j.geoderma.2009.08.002

Hamid R, Khan MA, Ahmad M, Ahmad MM, Abdin MZ, Musarrat J, Javed S (2013) Chitinases: An update. J Pharm Bioall Sci 5:21-29 doi:10.4103/0975-7406.106559

Hayden HL, Drake J, Imhof M, Oxley AP, Norng S, Mele PM (2010) The abundance of nitrogen cycle genes *amoA* and *nifH* depends on land-uses and soil types in South-Eastern Australia. Soil Biol Biochem 42:1774-1783 doi:10.1016/j.soilbio.2010.06.015

Haynes R (2012) Mineral nitrogen in the plant-soil system. Elsevier.

Isbell R (2002) The Australian soil classification vol 4. CSIRO Publishing, Collingwood, Vic, Australia.

IUSS Working Group WRB (2007) 'World reference base for soil resources 2006' World Soil Resources Report No. 103. ftp://ftp.fao.org/docrep/fao/009/a0510e/a0510e00.pdf. Accessed on 20th November, 2015

Jenkinson D, Ladd J (1981) Microbial biomass in soil: Measurement and turnover. In Paul EA and Ladd JN (Eds.), Soil Biochemistry, Volume 5. Marcel Dekker, New York, pp 415-471

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Methods 58:169-188 doi:http://dx.doi.org/10.1016/j.mimet.2004.04.006

Knowles R (1982) Denitrification. Microbiol Rev 46:43

Kuan H, Hallett P, Griffiths B, Gregory A, Watts C, Whitmore A (2007) The biological and physical stability and resilience of a selection of Scottish soils to stresses. Eur J Soil Sci 58:811-821 doi: 10.1111/j.1365-2389.2006.00871.x

Lee OO, Wang Y, Yang J, Lafi FF, Al-Suwailem A, Qian P-Y (2011) Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. ISME J 5:650-664 doi:10.1038/ismej.2010.165

Li M, Cao H, Hong Y, Gu J-D (2011) Spatial distribution and abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in mangrove sediments. Appl Microbiol Biotechnol 89:1243-1254 doi:10.1007/s00253-010-2929-0

Li P, Lang M (2014) Gross nitrogen transformations and related N_2O emissions in uncultivated and

cultivated black soil. Biol and Fertil Soils 50:197-206. doi:10.1007/s00374-013-0848-9

Liu H, Carvalhais L, Crawford M, Dang Y, Dennis P, Schenk P (2016a) Strategic tillage on a Grey Vertosol after fifteen years of no-till management had no short-term impact on soil properties and agronomic productivity. Geoderma 267C:146-155 doi:10.1016/j.geoderma.2016.01.002

Liu H, Rincon-Florez V, Crawford M, Dang Y, Carvalhais L, Paul D, Schenk P (2016b) One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol. Soil Till Res:191-199 doi:10.1016/j.still.2015.12.007

Llewellyn RS, D'Emden F (2010) Adoption of no-till cropping practices in Australian grain growing regions. Australian Government, Grains Research and Development Corporation. http://www.grdc.com.au/uploads/documents/GRDC_adoption_of_no-till.pdf. Accessed 1 December, 2016

Logsdon SD (2013) Depth dependence of chisel plow tillage erosion. Soil Till Res 128:119-124 doi:http://dx.doi.org/10.1016/j.still.2012.06.014

López-Garrido R, Madejón E, Murillo JM, Moreno F (2011) Soil quality alteration by mouldboard ploughing in a commercial farm devoted to no-tillage under Mediterranean conditions. Agric Ecosyst Environ 140:182-190 doi:http://dx.doi.org/10.1016/j.agee.2010.12.001

Melero S, Panettieri M, Madejon E, Macpherson HG, Moreno F, Murillo J (2011) Implementation of chiselling and mouldboard ploughing in soil after 8 years of no-till management in SW, Spain: Effect on soil quality. Soil Till Res 112:107-113 doi: 10.1016/j.still.2010.12.001

Miura T et al. (2015) Shifts in the composition and potential functions of soil microbial communities responding to a no-tillage practice and bagasse mulching on a sugarcane plantation. Biol and Fertil Soils 52: 307-322 doi: 10.1007/s00374-015-1077-1

Nagpure A, Choudhary B, Gupta RK (2014) Chitinases: in agriculture and human healthcare. Crit Rev Biotechnol 34:215-232 doi: 10.3109/07388551.2013.790874

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. Eur J Soil Sci 54:655-670 doi:10.1046/j.1351-0754.2003.0556.x

Naumoff DG, Dedysh SN (2012) Lateral gene transfer between the Bacteroidetes and Acidobacteria: The case of α-l-rhamnosidases. FEBS Lett 586:3843-3851 doi:http://dx.doi.org/10.1016/j.febslet.2012.09.005

Norton U, Bista P, Ghimire R, Norton JB (2014) US West: One-time summer tillage does not negate long-term benefits of no-till. Crops Soils mag 47:24-25 https://dl.sciencesocieties.org/publications/cns/pdfs/47/3/24. Accessed on 1 December, 2016

Oorts K, Merckx R, Gréhan E, Labreuche J, Nicolardot B (2007) Determinants of annual fluxes of CO_2 and N_2O in long-term no-tillage and conventional tillage systems in northern France. Soil Till Res 95:133-148 doi:http://dx.doi.org/10.1016/j.still.2006.12.002

Philippot L, Piutti S, Martin-Laurent F, Hallet S, Germon JC (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl Environ Microbiol 68:6121-6128 doi: 10.1128/AEM.68.12.6121-6128.2002

Philippot L, Tscherko D, Bru D, Kandeler E (2011) Distribution of high bacterial taxa across the

chronosequence of two alpine glacier forelands. Microb Ecol 61:303-312 doi:10.1007/s00248-010-9754-y

Raper R (2002) The influence of implement type, tillage depth, and tillage timing on residue burial. Trans ASAE 45:1281-1286 doi: 10.13031/2013.11056

Rincon-Florez VA, Dang YP, Crawford MH, Schenk PM, Carvalhais LC (2016) Occasional tillage has no effect on soil microbial biomass, activity and composition in Vertisols under long-term no-till. Biol Fertil Soils 52:191-202 doi: 10.1007/s00374-015-1066-4

Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl Environ Microbiol 68:3818-3829 doi: 10.1128/AEM.68.8.3818-3829.2002

Rotthauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704-4712

Sait M, Davis KE, Janssen PH (2006) Effect of pH on isolation and distribution of members of subdivision 1 of the phylum Acidobacteria occurring in soil. Appl Environ Microbiol 72:1852-1857 doi: 10.1128/AEM.72.3.1852-1857.2006

Sapkota T, Mazzoncini M, Bàrberi P, Antichi D, Silvestri N (2012) Fifteen years of no till increase soil organic matter, microbial biomass and arthropod diversity in cover crop-based arable cropping systems. Agron Sustain Dev 32:853-863 doi:10.1007/s13593-011-0079-0

Schloter M, Dilly O, Munch J (2003) Indicators for evaluating soil quality. Agric Ecosyst Environ 98:255-262 doi:10.1016/S0167-8809(03)00085-9

Sparling G, Pankhurst C, Doube B, Gupta V (1997) Soil microbial biomass, activity and nutrient cycling as indicators of soil health. Biological indicators of soil health:97-119

Triplett G, Dick WA (2008) No-tillage crop production: a revolution in agriculture! Agron J 100:S-153-S-165 doi:10.2134/agronj2007.0005c

Van Gestel M, Merckx R, Vlassak K (1993) Microbial biomass and activity in soils with fluctuating water contents. Geoderma 56:617-626 doi:10.1016/0016-7061(93)90140-G

Wakelin SA, Macdonald LM, Rogers SL, Gregg AL, Bolger TP, Baldock JA (2008) Habitat selective factors influencing the structural composition and functional capacity of microbial communities in agricultural soils. Soil Biol Biochem 40:803-813 doi:http://dx.doi.org/10.1016/j.soilbio.2007.10.015

Walker S (2012) Capturing opportunities and overcoming obstacles in Australian agronomy. Edited by I. Yunusa. Proceedings of 16th Australian Agronomy Conference 2012, 14-18 October 2012, Armidale, NSW. http://www.regional.org.au/au/asa/2012/plenary/8550_walkersr.htm

Wessén E, Hallin S, Philippot L (2010) Differential responses of bacterial and archaeal groups at high taxonomical ranks to soil management. Soil Biol Biochem 42:1759-1765 doi:10.1016/j.soilbio.2010.06.013

Wortmann C, Quincke J, Drijber R, Mamo M, Franti T (2008) Soil microbial community change and recovery after one-time tillage of continuous no-till. Agron J 100:1681-1686

doi:10.2134/agronj2007.0317

Wortmann CS, Drijber RA, Franti TG (2010) One-time tillage of no-till crop land five years post-tillage. Agron J 102:1302-1307 doi:10.2134/agronj2010.0051

Yan T, Fields MW, Wu L, Zu Y, Tiedje JM, Zhou J (2003) Molecular diversity and characterization of nitrite reductase gene fragments (nirK and nirS) from nitrate-and uranium-contaminated groundwater. Environ Microbiol 5:13-24 doi:10.1046/j.1462-2920.2003.00393.x

Yergeau E, Kang S, He Z, Zhou J, Kowalchuk GA (2007) Functional microarray analysis of nitrogen and carbon cycling genes across an Antarctic latitudinal transect. ISME J 1:163-179 doi:10.1038/ismej.2007.24

Yu Y, Zhang J, Chen W, Zhong W, Zhu T, Cai Z (2014) Effect of land use on the denitrification, abundance of denitrifiers, and total nitrogen gas production in the subtropical region of China. Biol Fertil Soils 50:105-113 doi: 10.1007/s00374-013-0839-x

Chapter 5 Effects of jasmonic acid signalling on the wheat microbiome differ between body sites

Overview

In chapters 2, 3 and 4 of this thesis, I tested the potential influence of strategic tillage on the soil microbial properties of long-term NT wheat field soils in the Australian northern grain growing regions. I hypothesised that microbiomes in soils with a long history of repeat wheat cultivation and NT practice harbour microbes that are well-adapted to wheat plants and that these may be influenced by different physiological states of wheat plants. Specifically, I tested in this chapter whether microbial communities in wheat field soils could be manipulated by defence hormone treated wheat plants. In chapter 5 and 6, wheat-mediated effects of two important plant hormones (jasmonate and salicylic acid) on wheat associated microbial communities were examined. Gaining knowledge on the dynamics of the microbial communities that live symbiotically with plants may facilitate highly productive, low-input agricultural systems in the future. The jasmonic acid (JA) signalling pathway is used by plants to defend themselves against necrotrophic pathogens and herbivorous insects, but it also plays a role during plant-beneficial rhizosphere microbe interactions. Recently, in *Arabidopsis*, activation of the JA signalling pathway has been shown to alter the composition of rhizosphere bacterial communities; however, this effect is yet to be investigated in other plants. Here, I examined the influence of the activation of JA signalling in wheat plants on the composition of bacterial communities associated with wheat shoot and root endosphere, and ectorhizosphere using 16S rRNA gene sequencing and other classical microbiological methods for measuring changes in soil microbial properties.

Highlights

- MeJA treatment altered the composition of endophytic microbial communities in wheat roots;
- Decreased microbial diversity was observed in endophytic roots;
- Bacterial communities in endophytic shoots or ectorhizosphere were not influenced by the elevated MeJA treatment;
- Ectorhizosphere CLPP or microbial activity were not influenced by MeJA treatments;
- Plant switched on JA signalling defence pathway may be associated with lower bacterial diversity.

Effects of jasmonic acid signalling on the wheat microbiome differ between body sites

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Abstract

Jasmonic acid (JA) signalling helps plants to defend themselves against necrotrophic pathogens and herbivorous insects and has been shown to influence the root microbiome of *Arabidopsis thaliana*. In this study, we determined whether JA signalling influences the diversity and functioning of the wheat (*Triticum aestivum*) microbiome and whether these effects are specific to particular parts of the plant. Activation of the JA pathway was achieved via exogenous application of methyl jasmonate and was confirmed by significant increases in the abundance of 10 JA-signalling-related gene transcripts. Phylogenetic marker gene sequencing revealed that JA signalling reduced the diversity and changed the composition of root endophytic but not shoot endophytic or ectorhizosphere bacterial communities. The total enzymatic activity and substrate utilisation profiles of ectorhizosphere bacterial communities were not affected by JA signalling. Our findings indicate that the effects of JA signalling on the wheat microbiome are specific to individual plant compartments.

1 Introduction

Plants are associated with diverse microbial communities that influence their health and nutrition[1].

These organisms are known collectively as the plant microbiome and could be used to more sustainably maintain or enhance global food security. To achieve this, ways to manipulate the structure of plant-associated microbial communities need to be identified. Recently, activation of the jasmonic acid (JA) plant defence pathway, which is involved in suppression of necrotrophic pathogens and herbivorous insects[2], was shown to alter the composition of the *Arabidopsis thaliana* root microbiome[3]. Activation of the JA signalling pathway increased the relative abundances of bacterial populations closely related to taxa that are reported to suppress phytopathogens and insects[3]. This suggests that when under attack plants may have evolved mechanisms to recruit symbionts that enhance their tolerance to biotic stress. Currently, however, it is not known whether the microbiome of other plant species are influenced by activation of the JA pathway, and whether these effects, if any, are also apparent in endophytic compartments of the host.

Given the intimate physical association between plants and endophytic symbionts, changes to the structure of endophytic communities may disproportionately influence host fitness. While JA signalling has been shown to restrict endophytic colonisation of rice (*Oryza sativa*) by incompatible strains of nitrogen-fixing *Azoarcus* bacteria[4] and suppress nodulation in *Lotus japonicas*[5], it remains unknown whether JA signalling influences the overall structure of endophytic microbiomes.

Wheat is one of the most important and widely grown crops worldwide. Despite this, the effects of JA signalling on wheat microbial communities have not been characterised. In this study, we used phylogenetic marker gene sequencing to determine whether activation of the JA pathway altered the diversity of bacterial and archaeal communities associated with the wheat ectorhizosphere and root and shoot endophytic environments. Increased JA signalling was achieved via exogenous application of methyl jasmonate (MeJA) and confirmed by quantification of JA-associated gene transcripts[6]. Lastly, we measured the total enzymatic activity and substrate utilisation profiles of microbial communities associated with the ectorhizosphere.

2 Results and Discussion

2.1 Activation of the JA signalling pathway

The transcriptional level of ten genes associated with activation of the wheat JA signalling pathway was quantified in shoot tissues 72 hours after MeJA application using real-time PCR (Fig. 1). Previously, we have demonstrated that these genes are strongly associated with the intensity of JA signalling[6]. Relative to the control, MeJA application led to significant increases in the abundance

of all gene transcripts as follows: *PR1.1* (+ 2.4 fold), *PR2* (+ 3.3 fold), *PR4a* (+ 2.3 fold), *PR5* (+ 3.0 fold), *PR9* (+ 8.0 fold), *WCI2* (+ 29.4 fold), *WCI3* (+ 25.4 fold), *CHI3* (+ 1.9 fold), *TaAOS* (+ 7.0 fold) and *LIPASE* (+ 14.3 fold) (Fig. 1). These results indicate that the MeJA treatment was successful in activating the JA signalling pathway.

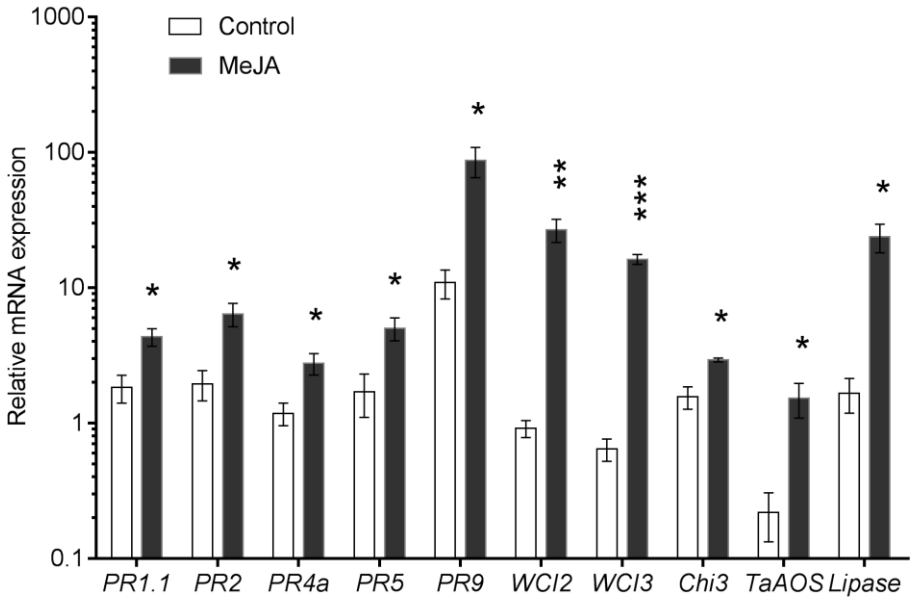

Fig. 1 The effect of MeJA application on the transcription of genes associated with the jasmonic acid (JA) signaling pathway in 10-day-old wheat seedlings. Asterisks indicate significant differences between control and MeJA treated plants ($P < 0.05^{*}$, $P < 0.01^{**}$, $P < 0.001^{***}$, two-tailed student's *t* test). Error bars represent standard errors of the means (n = 3).

2.2 Root and shoot endophytes

Relative to shoots, the diversity of root endophytic communities was richer (Sobs and Chao1) and more even (Simpson's Diversity Index) ($R^2 > 83\%$, $P < 0.001$) (Figs. 2 and S1). This is consistent with the fact that root endophytes typically derive from soil[7] and that shoot endophytes colonise either from root endophytic environments via the vascular tissue or enter via openings on stems and leaves[8,9]. The composition of endophytic communities also differed significantly between roots and shoots ($R^2 = 88.9\%$, $P = 0.002$; Figs. 3 and S2). Shoot endophytes were positively associated with members of the *Shewanella* (OTU 21-22) and a representative of the *Halomonas* (OTU 27) (Figs. 3 and S2). Root endophytes were positively associated with representatives of the *Streptomyces* (OTUs 11-14) and members of the *Actinosynnemataeae* (OTU 1) and *Glycomyces* (OTU 4) (Figs. 3 and S2). All of these taxa have previously been detected as endophytes in a wide-range of plant species. For example, representatives of the *Halomonas* have been observed in endophytic root and

shoot environments of: *Alopecurus aequalis*[10], *Typha domingensis*[11] and *Arthrocnemum macrostachyum*[12]. *Shewanella* spp. have been detected inside potato tubers[13], rice roots[14] and baby spinach leaves[15]. Actinobacteria, particularly *Streptomyces* spp., are frequently isolated from endophytic root and shoot environments of maize (*Zea mays* L.)[16], rice[17], tomato[18] and wheat[19-22] and members of the *Streptomycetaceae* are key components of endophytic communities in *Arabidopsis thaliana* roots[23,24].

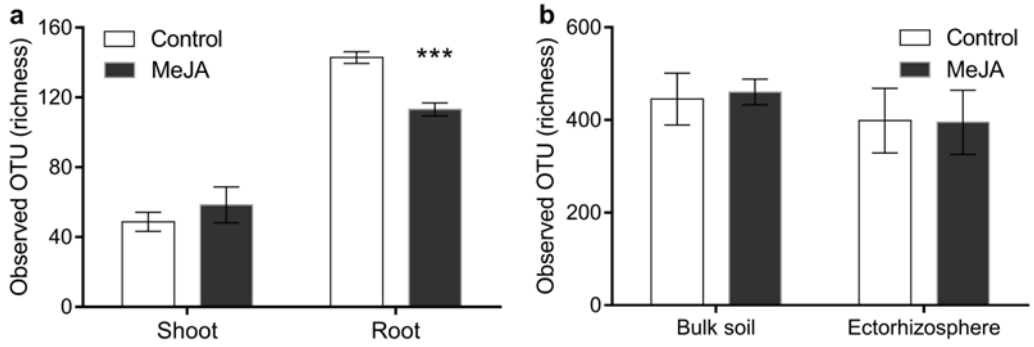

Fig. 2 The effect of MeJA treatment on the observed numbers of bacterial taxa (OTUs) associated with (a) wheat shoot and root endophytic environments, (b) bulk soil and the wheat ectorhizosphere. The asterisks indicate significant difference ($P < 0.001$) between treatments. All values were based on 1,250 rarefied sequences per sample. Error bars denote standard errors (n = 3).

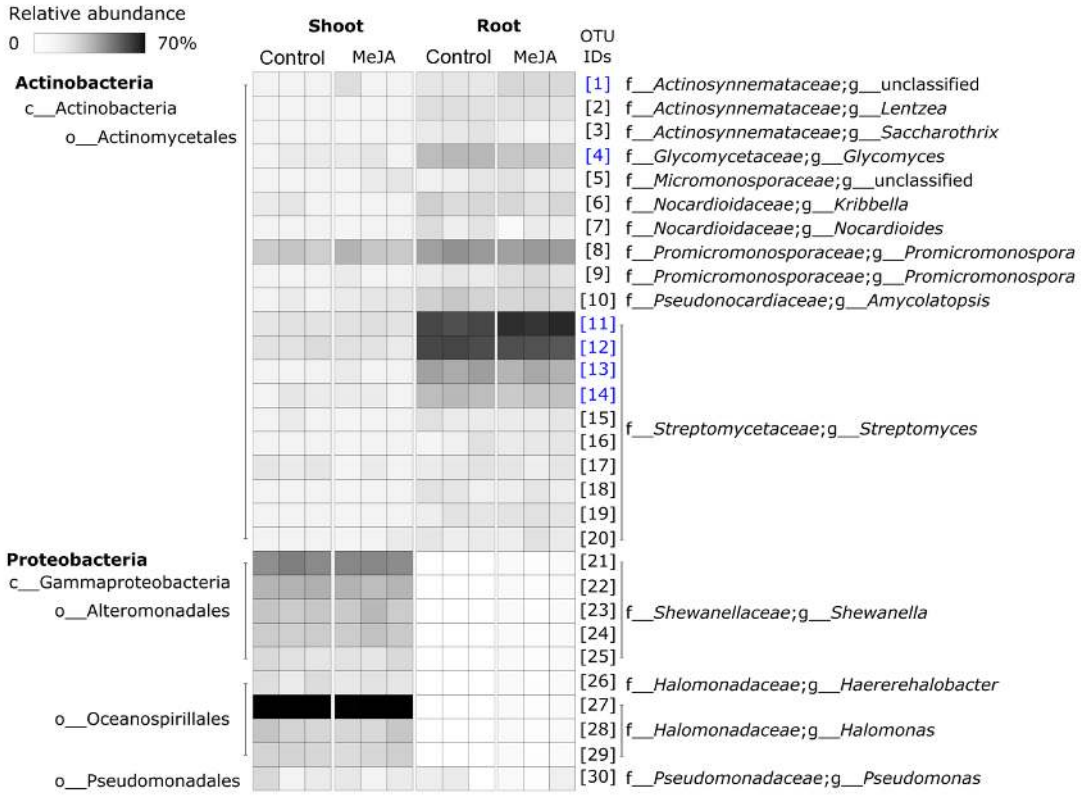

Fig. 3 Heatmap summarising variation in the composition of bacterial communities associated with wheat shoot and root endophytic environments with or without MeJA treatment. Each Operational Taxonomic Unit (OTU) has a unique numeric identifier shown in square brackets that is consistent with those shown in other figures.

2.3 The influence of JA signalling on the diversity of root and shoot endophytes

Activation of JA signalling led to a significant reduction in the richness ($P < 0.001$) and evenness ($P < 0.001$) of root, but not shoot, endophytic communities (Figs. 2 and S1). This novel finding may indicate that when under attack plants have evolved a mechanism to generally suppress microbial colonisation. However, absolute rather than relative abundances are needed to test this hypothesis. Previous studies have also reported no effects of JA signalling on the diversity of endophytes associated with aerial parts of plants[25]. Root endophytic communities may be more responsive to JA signalling because, relative to aboveground environments, soils harbour more organisms and, therefore, more potential attackers. Activation of JA signalling also led to a significant change in the composition of root, but not shoot, endophytic communities ($P = 0.011$; Figs. 3, 4 and S2). Relative to the control, MeJA treatment significantly increased the relative abundances of a *Actinosynnemataeae* (OTU 1) and a *Streptomyces* (OTU 11) population, and decreased the relative abundances of a *Glycomyces* (OTU 4) population and several members of the *Streptomyces* (OTUs 12-14) (Fig. 4). All of these taxa are members of the Actinobacteria, which include many populations that have been shown to promote plant growth, mobilise nutrients and suppress bacterial, fungal or viral phytopathogens[26-30]. For this reason, the observed changes in the relative abundances of actinobacterial populations in our study, may have had functional consequences for the host, which deserve further investigation in future studies.

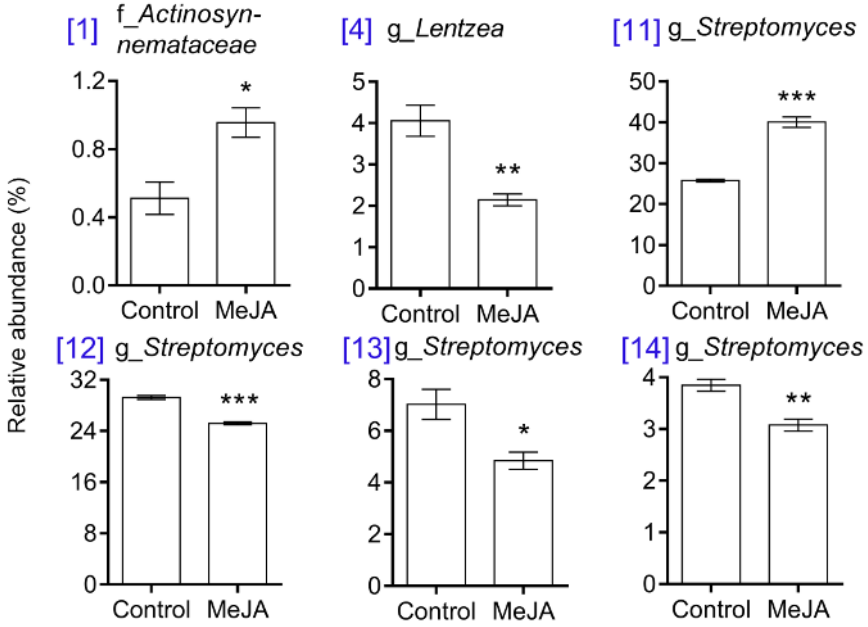

Fig. 3 Bacterial Operational Taxonomic Units (OTUs) associated with wheat root endophytic environments that were most strongly affected by MeJA treatment. The asterisks indicate significant differences between treatments ($P < 0.05^{*}$, $P < 0.01^{**}$, $P < 0.001^{***}$, two-tailed student's *t* test). Each OTU has a unique numeric identifier shown in square brackets that is consistent with those shown in other figures.

2.4 Ectorhizosphere and bulk soil microbial communities

Activation of the JA pathway did not significantly influence the richness, evenness or composition of bacterial communities associated with the ectorhizosphere or bulk soil ($P > 0.05$) (Figs. 2, 5 and S1). Likewise, activation of the JA pathway did not influence the total enzymatic activity or substrate utilisation profiles of microbial communities associated with ectorhizosphere or bulk soil (Fig. S3). While all previous studies indicate that JA signalling has no effect on the richness or evenness of ectorhizosphere bacterial communities[3,31], the effects on bacterial community composition are inconsistent. When grown in soil collected from areas where *A. thaliana* grows naturally, stimulation of the *A. thaliana* JA pathway led to a significant alteration in ectorhizosphere bacterial community composition[3]. However, when grown in 'non-native' soils, induction of the *A. thaliana* JA pathway had no effect on the composition of ectorhizosphere bacterial communities[31]. This suggests that JA pathway-mediated effects on ectorhizosphere bacterial communities may be influenced by soil type and the length of association between a particular plant genotype and soil. The soil selected in our study had a long cropping history of wheat but we did not detect any effects on ectorhizosphere bacterial communities within three days of JA signalling. This does not rule out

the possibility that effects may become apparent over longer time periods or for plants grown in other soils.

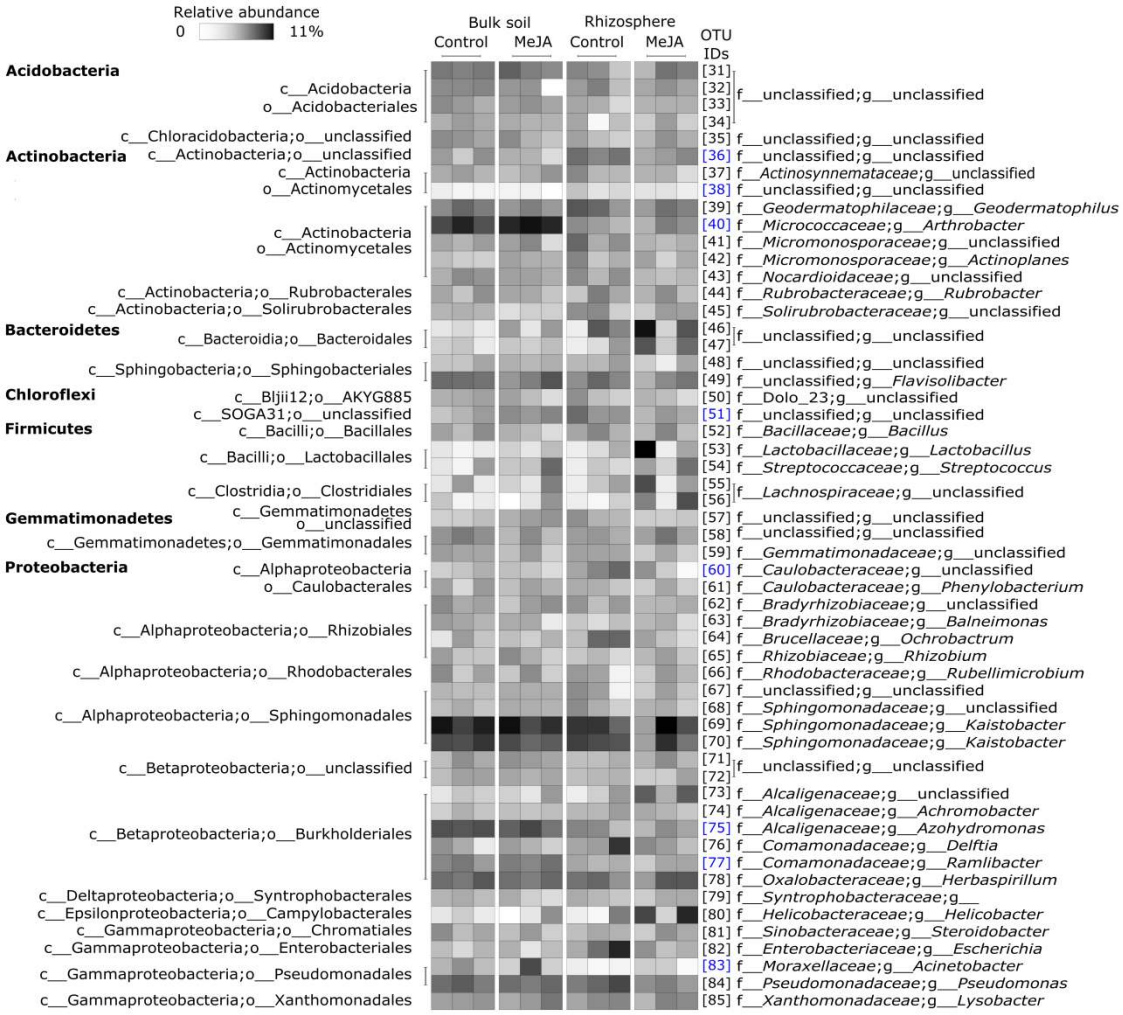

Fig. 4 Heatmap summarising variation in the composition of bacterial communities between bulk soil and the wheat root ectorhizosphere with or without MeJA treatment. Each Operational Taxonomic Unit (OTU) has a unique numeric identifier shown in square brackets that is consistent with those shown in other figures. OTUs highlighted in blue differ between bulk soil and the wheat ectorhizosphere ($P < 0.05$).

As observed in many studies[32,33], the composition of bacterial communities in the ectorhizosphere differed from those of those associated with bulk soil (R^2 = 13.3%, P = 0.048; Figs. 5 and S4). The ectorhizosphere was associated with larger relative abundances Actinomycetales (OTU 36, 38), Chloroflexi (OTU 51) and Caulobacteraceae populations (OTU 60), while bulk soil was positively associated with members of *Arthrobacter* (OTU 40), *Azohydromonas* (OTU 75), *Acinebacter* (OTU 83) and *Ramlibacter* (OTU 77) (Figs. 5 and S4). Relative to bulk soil, the ectorhizosphere was also associated with more microbial enzyme activity ($P < 0.001$; Fig. S3). Bacterial community richness

and evenness (Figs. 1 and S1) and microbial substrate utilisation profiles (Fig. S3), however, were similar between ectorhizosphere and bulk soil samples.

3 Conclusion

Our study demonstrates that activation of JA signalling in wheat reduces the diversity and changes the composition of bacterial communities in endophytic roots but not in shoots or in the ectorhizosphere. Most of the root endophytic populations that became more abundant in response to JA signalling were closely related to taxa previously reported to promote plant growth, mobilise nutrients or suppress bacterial, fungal or viral phytopathogens[26-30]. This suggests that JA signalling may select for microbial symbionts that enhance host stress tolerance.

4 Materials and Methods

4.1 Plant growth conditions and experimental design

Wheat (*Triticum aestivum*) seeds (Crusader variety) were pre-germinated on a moist filter paper in a petri-dish for 36 h and then planted in 30-well punnet trays with three seeds per well (Fig. S5). Plants were grown in soil collected from 0-10 cm depth in a long-term wheat paddock in Condamine, Queensland, Australia (26.90°S, 149.64°E). Key physicochemical characteristics of this soil are summarised in Table S1. The soil was a mesotrophic effervescent Brown Sodosol developed on Cainozoic sand plains and had been under no-till management for 19 years. This paddock has a long cropping history of wheat and the previous crop on this soil was also wheat. The soil contained 25% clay, 14% silt and 61% sand and was homogenised prior to planting using a 2.4 mm sieve. Two additional trays were filled with soil but were not planted (Fig. S5). All trays were transferred to a controlled environment chamber (Percival Scientific, Boone, IA, USA) at 20 °C with a photoperiod of 12 h and light intensity of 150 mmol m^{-2} s^{-1}. Throughout the experiment, the plants were watered once per two days with an amount ~10 mL per well, and the positions of the trays within the growth chamber were changed on a daily basis.

After 10 days (two-leaf stage), the JA signalling pathway was activated by exogenously applying methyl jasmonate (MeJA) as previously described[3]. Briefly, 300 µL, 0.5% (v/v ethanol) of MeJA was applied on a cotton ball attached to the lid of the tray to create an atmosphere containing 0.025 µL MeJA L^{-1}. The tray was then immediately sealed with tape and enclosed in two sealed transparent plastic bags. The same procedure was repeated for the control plants but MeJA was

omitted and 300 µL of ethanol which was the solvent used to prepare MeJA solution was applied to the cotton ball. To determine whether MeJA led to any direct effects on soil microorganisms one of the unplanted trays was treated with 300 µl MeJA solution and compared to another tray that was treated with 300 µl ethanol. We included three replicates per treatment. Each plant replicate comprised a pool of 30 plants.

4.2 Sample collection

Bulk soil and ectorhizosphere samples: All samples were collected 72 h post-MeJA treatment (Fig. S5). For bulk soil samples, soil was collected in sterile tubes and then stored at -80ºC until further processing. For ectorhizosphere soil samples, roots were carefully removed from each pot, excess soil was removed by shaking and that remaining closely adhered to the roots was considered to be ectorhizosphere soil[3]. For DNA extraction, ectorhizosphere soil was recovered by shaking roots in sterile 50 ml tubes each containing 25 ml sterile phosphate buffer (Na_2HPO_4 7.1 g, $NaH_2PO_4 \cdot H_2O$ 4.4 g, amended to 820 mL, pH 7.0, 0.1 M) for five min at 250 rpm. After shaking, roots were transferred to new tubes and ectorhizosphere soil was pelleted by centrifugation at 12,000 g for 3 min then transferred to -80°C storage until further processing. For MicroResp™ (James Hutton Institute, Invergowrie, Scotland, UK)[41], ectorhizosphere soil was physically separated from roots using sterile gloves.

Root and shoot endophytic samples: After removal of ectorhizosphere soil, root tissues were washed with distilled water and 0.1% Silwet L-77 in phosphate buffer three times[36], sonicated at 20 kHz for five min to remove rhizoplane microorganisms[24], washed in sterile phosphate buffer, air dried, ground in liquid nitrogen and then stored at -80 °C for DNA extraction. For shoots, half of the tissues were immediately submerged in liquid nitrogen and stored at -80°C for RNA extraction (Fig. S5). The other half were washed with 0.1% Silwet L-77 in phosphate buffer three times, surface sterilised using 0.5% (v/v) hypochlorite for two min, air dried, ground in liquid nitrogen and then stored at -80 °C for DNA extraction.

4.3 Quantification of JA signalling pathway-related transcripts

Total RNA was extracted from wheat shoots using the SV Total RNA Isolation Kit (Promega) according to the manufacturer's recommendations. The cDNA was synthesised by reverse transcription of 1.5 µg of total RNA using the Superscript III kit (Life Technologies) and both random hexamers and oligo dT primers. Quantitative real-time PCR (qRT-PCR) assays were

performed on a ViiA™ 7 sequence detection system (Applied Biosystems, USA). Ten JA defence-related genes in wheat, namely *PR1.1*, *PR2*, *PR4a*, *PR5*, *PR9*, *WCI2*, *WCI3*, *CHI3*, *TaAOS* and *LIPASE* were examined for gene expression in shoots. Primer sequences are shown in Table S2. The wheat 18S rRNA gene was used as an internal reference gene for normalisation. PCR conditions and the relative expression of each target gene was investigated as previously described[6].

4.4 DNA extraction and 16S rRNA gene amplification and sequencing

For bulk soil and rhizosphere samples, DNA was extracted from two grams of soil using the Power Soil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's recommendations. For root and shoot samples, DNA was extracted from 0.2 g plant tissue using a CTAB method[36]. Extracted DNA was then quantified using a Qubit™ fluorometer with Quant-iT dsDNA BR Assay Kit (Invitrogen) and normalised to 1 ng μL^{-1} and 20 ng μl^{-1} for soil and plant extracts, respectively.

Bacterial 16S rRNA genes were amplified by PCR with 803F (5'-ATT AGA TAC CCT GGT AGT C-3') and 1392wR (5'-ACG GGC GGT GWG TRC-3') for bulk soil and rhizosphere samples. PCR primers pairs of 799F (5'-AAC MGG ATT AGA TAC CCK G-3') and 1193R (5'-ACG TCA TCC C CA CCT TCC-3') were used for the amplifications of root and shoot endophytic bacteria. The primer pair 799F and 1193R spans the hypervariable regions V5-V6-V7 of the 16S rRNA gene and amplifies preferentially archaeal and bacterial DNA and avoids amplification of plant eukaryotic DNA[37]. For the above two primer pairs, B adaptor (5'-CCT ATC CCC TGT GTG CCT TGG CAG TC-3') was linked to a key (TCAG) and connected to template specific forward primers. An adaptor (3'-CCA TCT CAT CCC TGC GTG TCT CCG AC-5') was linked to key (TCAG) and sample specific MID, and then was connected to template specific reverse primer. The MID sequence contained a five-base barcode sequence positioned between the primer sequence and the adapter.

Bacterial and archaeal 16S rRNA genes in soil and endophytic roots and shoots were amplified by PCR which was carried out in a 25 µL reaction containing 14.75 µL ultra-pure water, 5.0 µL 5×phire buffer, 1.25 µL 10 µM dNTPs, 1.25 µL 10 µM forward primer, 1.25 µL 10 µM reverse primer, 0.5 µL phire® hot start II, and 1 µL of DNA template (1 and 20 ng for soil and plant samples, respectively). PCR conditions were 30 s at 98°C for initial denaturation, 29 cycles of 10 s at 98°C, 30 s at 56°C for the annealing step and 45 s at 72 °C, with 7 min of 72 °C for final extension step.

Amplicons of the 16S rRNA gene (~400 bp) generated by PCR primers 799F and 1193R were

excised from an agrose gel (1.5%) and were further purified using a Wizard® SV Gel and PCR Clean-Up System (Promega). After purification, amplification products were quantified using a Qubit™ fluorometer with Quant-iT dsDNA HS Assay Kits (Invitrogen), normalised to 25 ng μL^{-1} per sample and then pooled for 454 pyrosequencing. Sequencing was performed by Macrogen (Seoul, Korea).

4.5 Processing of sequence data

Data were processed as described previously[37]. Briefly, sequences were quality filtered and dereplicated using the QIIME script split_libraries.py with the homopolymer filter deactivated[38], checked for chimeras against the GreenGenes database (October 2013 release) using UCHIME ver. 3.0.617[39], homopolymer error corrected using Acacia[40] and then subjected to the following procedures using QIIME: (1) OTUs were picked at 97% similarity, (2) OTU representative sequences were assigned GreenGenes (October 2013) taxonomy using BLAST, and then (3) tables with the abundance of different operational taxonomic units (OTUs) and their taxonomic assignments in each sample were generated. The number of reads was rarefied to 1,250 per sample to allow comparisons of diversity without the bias of uneven sampling effort. The mean number of OTUs (observed richness) and Simpson's Diversity Index values corresponding to 1,250 sequences per sample were calculated using QIIME.

4.6 Microbial community activity

Community-level physiology profiles (CLPPs) were generated by characterising the induced respiratory responses of microorganisms associated with 0.4 g of each soil sample to 20 substrates using MicroResp™,[41] as described in Liu et al.[42]. The substrates included carboxylic acids (citric acid, methyl pyruvate, oxalic acid, D+galacturonic acid and succinic acid), carbohydrates (beta-d-fructose, D-(+)-trehalose, D-glucose, L-malic acid, D-xylose, mannitol, L-(+) Arabinose, cellulose), amino acids (L-alanine, gamma-aminobutyric acid, L-arginine, L-Asparagine), urea, uric acid and tween 40. Milli-Q water was added to controls.

Fluorescein diacetate (FDA) hydrolysis assays were used to provide a measure of total microbial enzyme activity and were performed as described by Green et al.[43].

4.7 Statistical analyses

The effect of MeJA treatment on enzyme activities and the richness and equitability of bacterial

communities was investigated using ANOVA. Differences in transcript abundances were assessed using two tailed *t*-tests. The effects of MeJA treatment on the composition of bacterial communities and on substrate utilisation patterns were investigated using Permutational Multivariate Analysis of Variance (PERMANOVA). PERMANOVA was performed using Hellinger transformed OTU abundances. Differences in the abundances of individual OTUs between treatments were identified using ANOVA with posthoc Tukey's HSD tests. All analyses were implemented using R (version 2.12.0). Differences in the composition of microbial communities or the utilisation of substrates between samples were visualised using principal component analysis (PCA) and/or heatmaps.

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Author contributions

H. L., P. M. S., L. C. C and P.G.D. conceived and designed the study. H. L. and L. C. C. performed experiments. P.G.D. and H. L. analysed the data and wrote the manuscript.

Competing financial interests

The authors declare no conflict of interest.

Supplementary information

Supplementary information is associated with the online version of this paper.

Data accessibility

The 16S rRNA amplicon sequences associated with this study have been deposited in the NCBI SRA under accession: PRJNA351276.

References

1. Berendsen, R. L., Pieterse, C. M. & Bakker, P. A. The rhizosphere microbiome and plant health. *Trends Plant Sci.* **17**, 478-486 (2012).
2. Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic

pathogens. *Annu. Rev. Phytopathol.* **43**, 205-227 (2005).

3. Carvalhais, L. C. *et al.* Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. *PLoS One* **8**, e56457 (2013).

4. Miché, L., Battistoni, F., Gemmer, S., Belghazi, M. & Reinhold-Hurek, B. Upregulation of jasmonate-inducible defense proteins and differential colonization of roots of *Oryza sativa* cultivars with the endophyte *Azoarcus* sp. *Mol. Plant Microbe In.* **19**, 502-511, (2006).

5. Nakagawa, T. & Kawaguchi, M. Shoot-applied MeJA suppresses root nodulation in *Lotus japonicus*. *Plant cell physiol.* **47**, 176-180 (2006).

6. Liu, H., Carvalhais, L. C., Kazan, K. & Schenk, P. M. Development of marker genes for jasmonic acid signaling in shoots and roots of wheat. *Plant Signal. Behav.* **11**, e1176654 (2016).

7. Compant, S., Clément, C. & Sessitsch, A. Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* **42**, 669-678 (2010).

8. Chi, F. *et al.* Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl. Environ. Microb.* **71**, 7271-7278 (2005).

9. Vorholt, J. A. Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* **10**, 828-840 (2012).

10. Peng, A., Liu, J., Gao, Y. & Chen, Z. Distribution of endophytic bacteria in *Alopecurus aequalis* Sobol and *Oxalis corniculata* L. from soils contaminated by polycyclic aromatic hydrocarbons. *PloS One* **8**, e83054 (2013).

11. Shehzadi, M. *et al.* Enhanced degradation of textile effluent in constructed wetland system using *Typha domingensis* and textile effluent-degrading endophytic bacteria. *Water Res.* **58**, 152-159 (2014).

12. Mora-Ruiz, M. d. R., Font-Verdera, F., Orfila, A., Rita, J. & Rosselló-Móra, R. Endophytic microbial diversity of the halophyte *Arthrocnemum macrostachyum* across plant compartments. *FEMS Microbiol. Ecol.* **92**, fiw145 (2016).

13. Sturz, A., Christie, B. & Matheson, B. Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Can. J. Microbiol.* **44**, 162-167 (1998).

14. Hardoim, P. R., Hardoim, C. C. P., van Overbeek, L. S. & van Elsas, J. D. Dynamics of seed-borne rice endophytes on early plant growth stages. *PLoS One* **7**, e30438 (2012).

15. Jackson, C. R., Randolph, K. C., Osborn, S. L. & Tyler, H. L. Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiol.* **13**, 1-12 (2013).

16. Araújo, J. M. d., Silva, A. C. d. & Azevedo, J. L. Isolation of endophytic actinomycetes from roots and leaves of maize (*Zea mays* L.). *Braz. Arch. Biol. Technol.* **43**, 0-0 (2000).

17. Kampapongsa, D. & Kaewkla, O. Biodiversity of endophytic actinobacteria from jasmine rice (*Oryza sativa* L. KDML 105) grown in Roi-Et Province, Thailand and their antimicrobial activity against rice pathogens. *Ann. Microbiol.* **66**,587-595 (2015).

18. Cao, L., Qiu, Z., You, J., Tan, H. & Zhou, S. Isolation and characterization of endophytic Streptomyces strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. *Lett. Appl. Microbiol.* **39**, 425-430 (2004).

19. Sadrati, N., Daoud, H., Zerroug, A., Dahamna, S. & Bouharati, S. Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (*Triticum durum*). *J. Plant Prot. Res.* **53**, 128-136 (2013).

20. Jog, R., Pandya, M., Nareshkumar, G. & Rajkumar, S. Mechanism of phosphate solubilization and antifungal activity of *Streptomyces* spp. isolated from wheat roots and rhizosphere and their application in improving plant growth. *Microbiology* **160**, 778-788 (2014).

21. Durán, P. *et al.* Endophytic bacteria from selenium-supplemented wheat plants could be useful for plant-growth promotion, biofortification and *Gaeumannomyces graminis* biocontrol in wheat production. *Biol. Fert.Soils* **50**, 983-990 (2014).

22. Coombs, J. T. & Franco, C. M. Isolation and identification of actinobacteria from surface-sterilized wheat roots. *Appl. Environ. Microb.* **69**, 5603-5608 (2003).

23. Lundberg, D. S. *et al.* Defining the core *Arabidopsis thaliana* root microbiome. *Nature* **488**, 86-90 (2012).

24. Bulgarelli, D. *et al.* Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* **488**, 91-95 (2012).

25. Kniskern, J. M., Traw, M. B. & Bergelson, J. Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on Arabidopsis thaliana. *Mol. Plant Microbe In.* **20**, 1512-1522 (2007).

26. Passari, A. K. *et al. In vitro* and *in vivo* plant growth promoting activities and DNA fingerprinting of antagonistic endophytic actinomycetes associates with medicinal plants. *Plos One* **10**, e0139468 (2015).

27. Xue, L. *et al.* Isolation and evaluation of rhizosphere actinomycetes with potential application for biocontrol of Verticillium wilt of cotton. *Crop Prot.* **43**, 231-240 (2013).

28. Mahajan, G. B. & Balachandran, L. Antibacterial agents from actinomycetes-a review. *Front. Biosci. (Elite edition)* **4**, 240-253 (2011).

29. El-Tarabily, K., Nassar, A., Hardy, G. S. J. & Sivasithamparam, K. Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. *J. Appl. Microbiol.* **106**, 13-26 (2009).

30. Zeng, Q. *et al.* A new nematicidal compound produced by *Streptomyces albogriseolus* HA10002. *A. Van. Leeuw. J. Microb.* **103**, 1107-1111 (2013).

31. Doornbos, R. F., Geraats, B. P., Kuramae, E. E., Van Loon, L. & Bakker, P. A. Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. *Mol. Plant Microbe In.* **24**, 395-407 (2011).

32. Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M. & Hurek, T. Roots shaping their microbiome: global hotspots for microbial activity. *Annu. Rev. Phytopathol.* **53**, 403-424 (2015).

33. Edwards, J. *et al.* Structure, variation, and assembly of the root-associated microbiomes of rice. *P. Natl. Acad. Sci.* **112**, E911-E920 (2015).

34. Lebeis, S. L. *et al.* Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* **349**, 860-864 (2015).

35. Horton, M. W. *et al.* Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. *Nat. Commun.* **5** (2014).

36. Porebski, S., Bailey, L. G. & Baum, B. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep.* **15**, 8-15 (1997).

37. Dennis, P. G. *et al.* Spatial uniformity of microbial diversity in a continuous bioelectrochemical system. *Bioresource Technol.* **129**, 599-605 (2013).

38. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335-336 (2010).

39. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200 (2011).

40. Bragg, L., Stone, G., Imelfort, M., Hugenholtz, P. & Tyson, G. W. Fast, accurate error-correction of amplicon pyrosequences using Acacia. *Nat. Methods* **9**, 425-426 (2012).

41. Campbell, C. D., Chapman, S. J., Cameron, C. M., Davidson, M. S. & Potts, J. M. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. *Appl. Environ. Microb.* **69**, 3593-3599 (2003).

42. Liu, H. *et al.* One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol. *Soil Till. Res.* **158**, 91-99 (2016).

43. Green, V., Stott, D. & Diack, M. Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. *Soil Biol. Biochem.* **38**, 693-701 (2006).

Supplementary Information

For "Effects of jasmonic acid signalling on the wheat microbiome differ between body sites"

Contents:

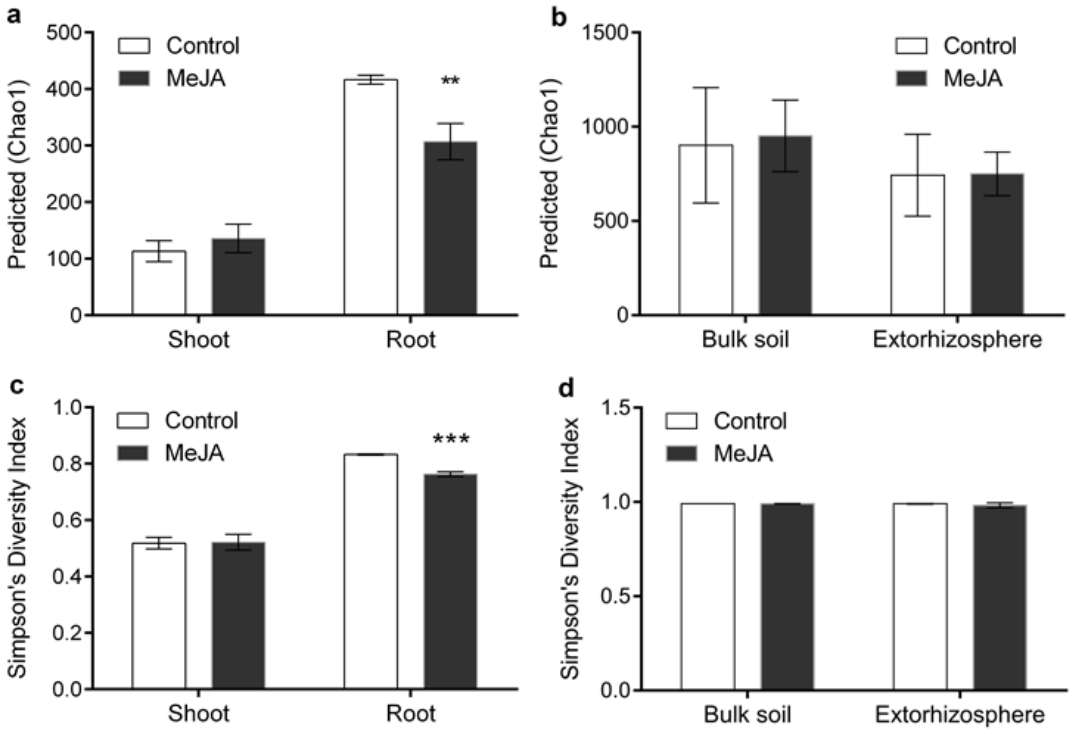

Fig. S1 The effect of MeJA treatment on the (a, b) predicted richness (Chao1) and (c, d) evenness (Simpson's Diversity Index) of bacterial communities associated with (a, c) wheat shoot and root endophytic environments, and (b, d) bulk soil and the wheat ectorhizosphere. The asterisks indicate significant differences between treatments: $P < 0.01$ $^{(**)}$, $P < 0.001$ $^{(***)}$. All values were based on 1,250 rarefied sequences per sample. Error bars denote standard errors (n = 3).

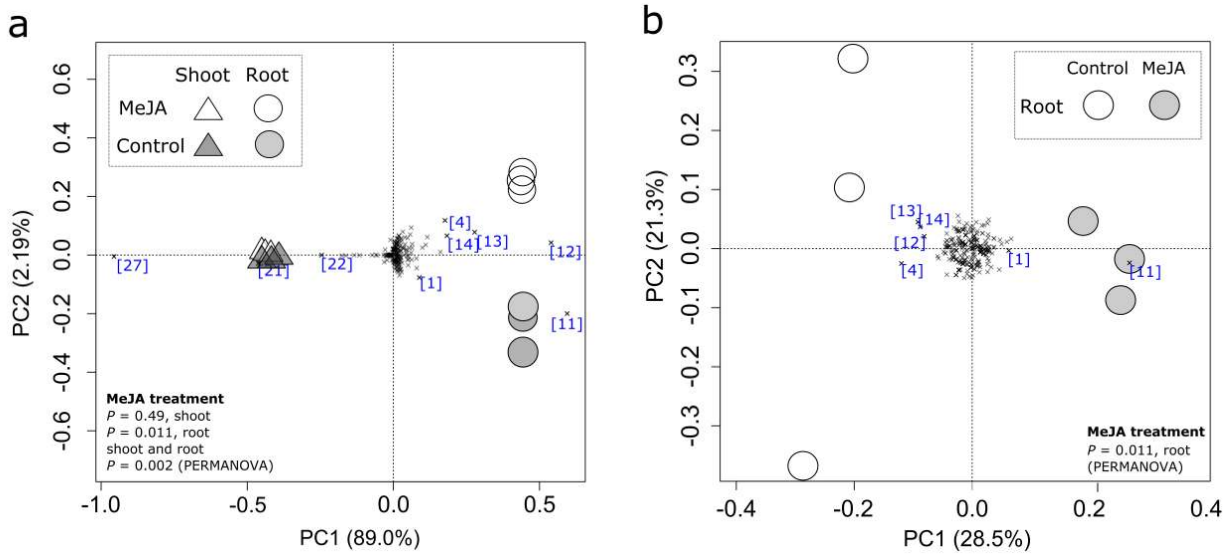

Fig. S2 Principal Component Analysis (PCA) summarising compositional differences in (a) wheat shoot and root endophytic microbial communities; or (b) just wheat endophytic root microbial communities. The numbers in square brackets represent OTU ids and correspond to those shown in Figure 2.

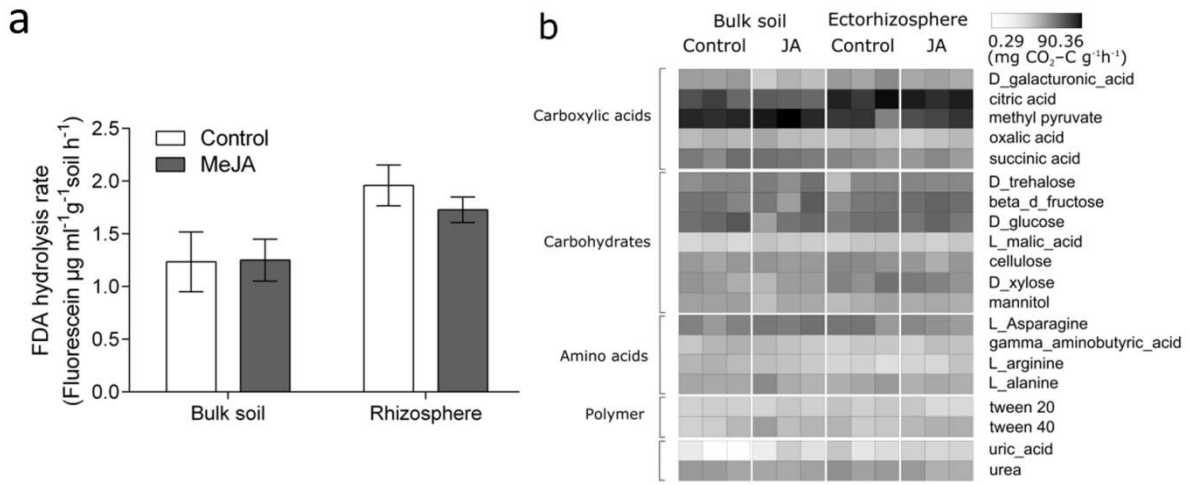

Fig. S3 The effect of MeJA treatment on (a) total microbial enzyme activity as indicated by FDA hydrolysis rates, and (b) substrate utilisation profiles based on Microresp^{TM} assays. Error bars denote the standard errors of the mean (n = 3).

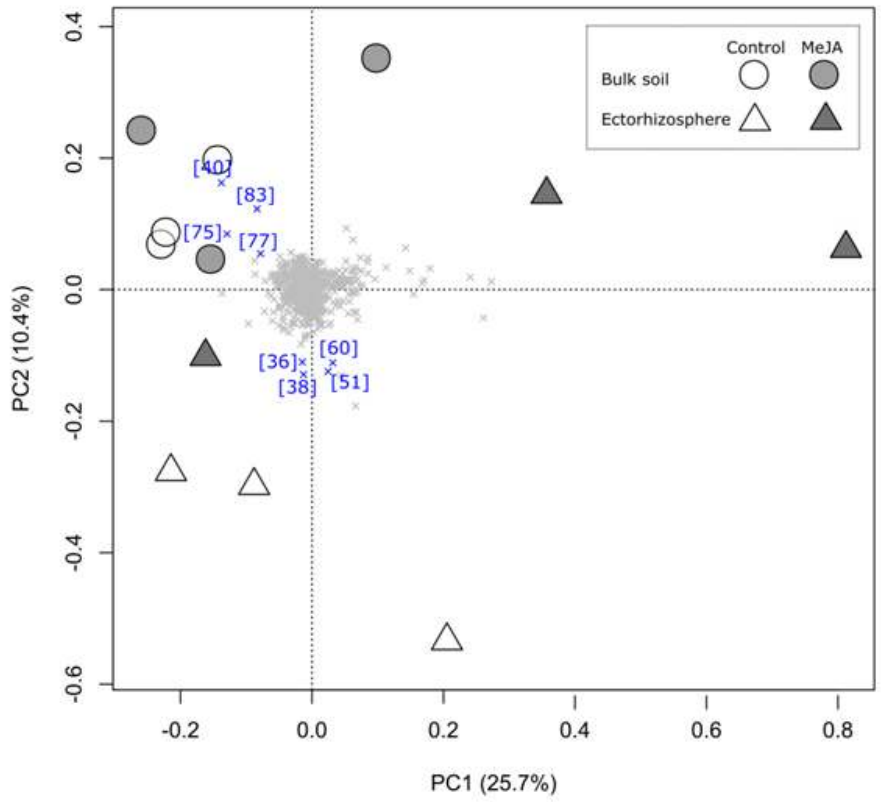

Fig. S4 Principal Component Analysis (PCA) summarising variation in the composition of bacterial communities associated with the wheat ectorhizosphere and bulk soil environments. The numbers shown in square brackets represent OTU ids and correspond to those shown in Figure 5.

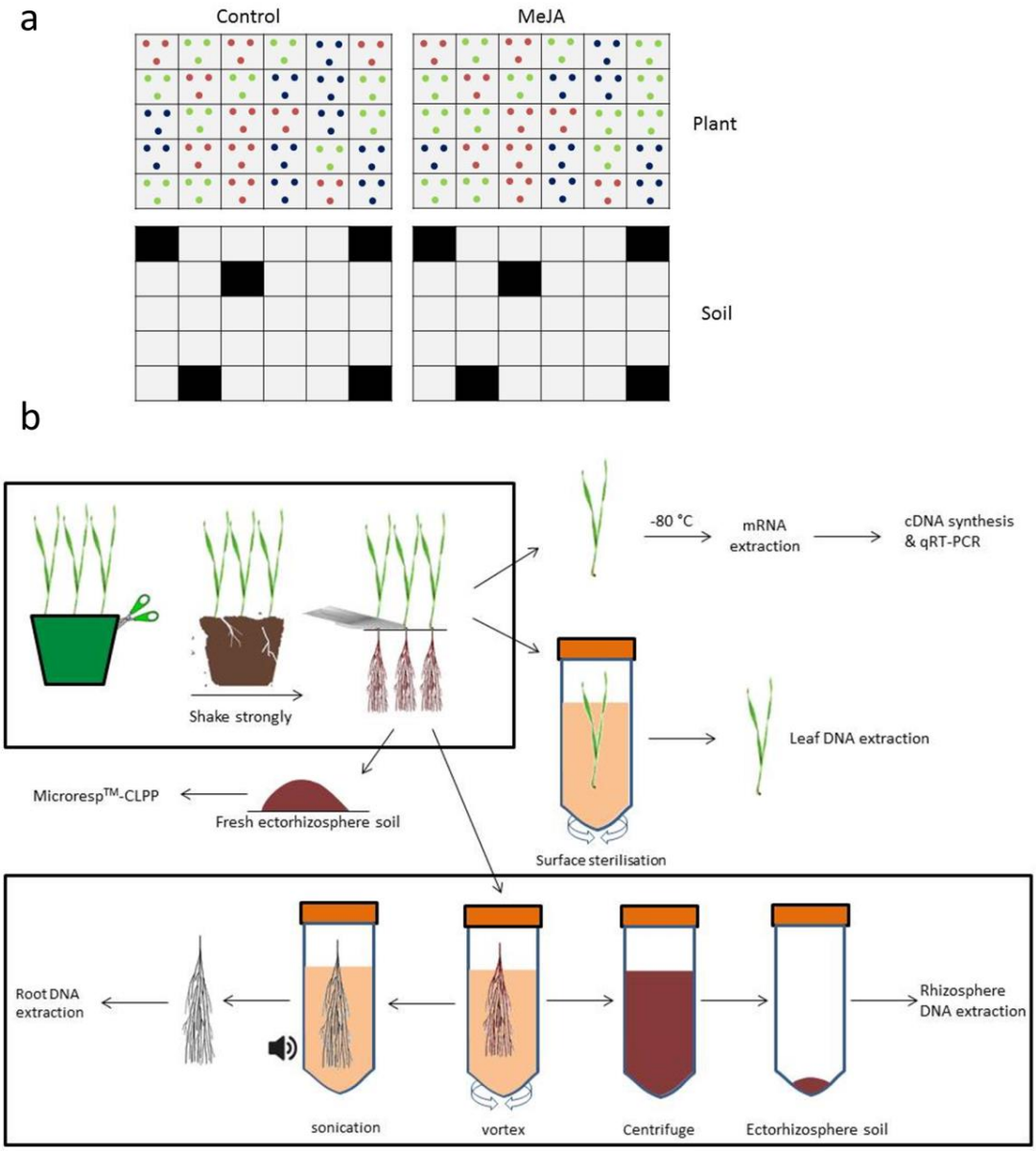

Fig. S5 Experiment design (a) and sampling (b). In panel a the four grids represent punnet trays. Half are used for plants which are represented by red, green and blue dots that indicate different bioreplicates. The other trays were used for bulk soil and were only partly filled as indicated by the black squares. Panel b shows the sampling methods, which correspond to the discriptions in the Materials and Methods of this study. Breifly, ectorhizosphere soil was collected using the 'pull and shake' method. The sampling procedures for the ectorhizosphere soil, and the wheat root and shoot tissues as well as surface sterilisation methods are detailed in the Materials and Methods.

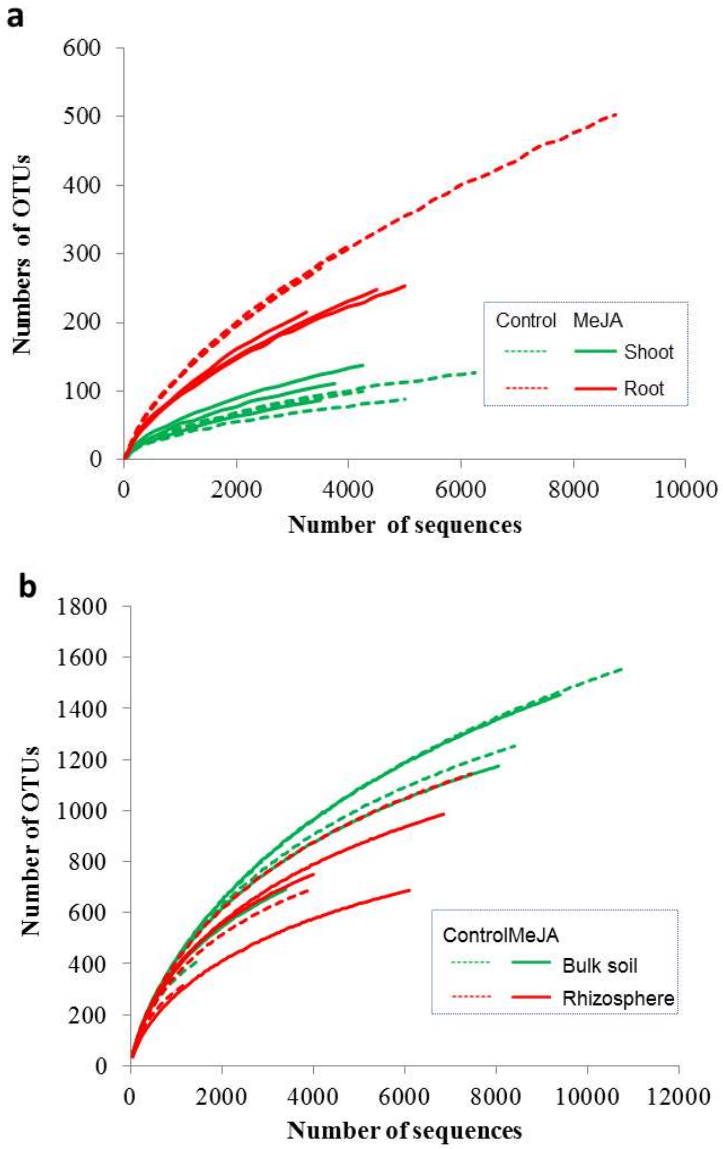

Fig. S6 Rarefaction curves showing that the communities were not exhaustively sampled.

Table S1 Soil physicochemical characteristics

Soil parameter	Value
pH	7.4
Organic carbon content (%)	1.28
Total carbon content (%)	1.36
Total nitrogen (%)	0.18
Total Al concentration (ppm)	30590
Total Ca concentration (ppm)	1781
Total Cu concentration (ppm)	17
Total Fe concentration (ppm)	21013
Total K concentration (ppm)	1666
Total Mg concentration (ppm)	154
Total Mn concentration (ppm)	1936
Total Na concentration (ppm)	933
Total P concentration (ppm)	278
Total S concentration (ppm)	355
Total Zn concentration (ppm)	46

Table S2 Primer sequences used for real-time PCR assays designed to confirm methyl-jasmonate induced activation of JA signalling in wheat.

Accession	Gene	Forward primer sequence	Reverse primer sequence	Gene description
[1]AF159369	*18S*	CAAAGCAAGCCTACGCTCT	ATACGAATGCCCCCGACT	*Haematococcus pluvialis* 18S ribosomal RNA gene
[2]AJ007348	*PR1.1*	CTGGAGCACGAAGCTGCAG	CGAGTGCTGGAGCTTGCAGT	*PR1* (basic), pathogenesis-related protein 1
[2]Y18212	*PR2*	CTCGACATCGGTAACGACCAG	GCGGCGATGTACTTGATGTTC	beta-1,3-endoglucanase
[2]AJ006098	*PR4a*	CGAGGATCGTGGACCAGTG	GTCGACGAACTGGTAGTTGACG	*wheatwin 1-2* gene
[2]AF442967	*PR5*	ACAGCTACGCCAAGGACGAC	CGCGTCCTAATCTAAGGGCAG	WAS3a thaumatin-like protein
[2]X56011	*PR9*	GAGATTCCACAGATGCAAACGAG	GGAGGCCCTTGTTTCTGAATG	wheat peroxidase
[1]AB029936	*CHI3*	GACCTCCTTGGCGTCAGCTA	TGCATGTCTTCTCGCATCATATAGTC	class 1b neutral chitinase
[2]U32428	*WCI2*	TAGGAACTGGAACTTCACCGAGC	GGTAGTCCTTGATGTGCAGCGAC	wheat chemically induced (*WCI*) gene, Lipoxygenase (Fragment)
[2]U32429	*WCI3*	AAAGTTGGTCTTGCCACTGACTG	TCGACAAAGCACTTCTGGATTTC	wheat chemically induced (*WCI*) gene, sulfur-rich/thionin-like protein
[1]AY196004	*TaAOS*	TCCCGAGAGCGCTGTTTAAA	GACGATTGACGGCTGCTATGA	*Triticum aestivum* allene oxide synthase
[3]TaBs117A2	*LIPASE*	CACAAAATATCGACCCACCAC	ACTGGGTATTCGTCTGTCAGC	wheat lipase

[1]Liu, H., Carvalhais, L. C., Kazan, K., Schenk, P. M. Development of marker genes for jasmonic acid signaling in shoots and roots of wheat. *Plant Signal. Behav.* 11(5), e1176654 (2016).

[2]Desmond O. J., Edgar, C. I., Manners, J. M., Maclean, D. J., Schenk, P. M., Kazan, K. Methyl jasmonate induced gene expression in wheat delays symptom development by the crown rot pathogen *Fusarium pseudograminearum*. *Physiol. Mol. Plant P.* 67(3), 171–179 (2006)

[3]Lu, Z., Gaudet, D., Puchalski, B., Despins, T., Frick, M., Laroche, A. Inducers of resistance reduce common bunt infection in wheat seedlings while differentially regulating defence-gene expression. *Physiol. Mol. Plant P.* 67(3), 138-148 (2006).

Chapter 6 Effects of salicylic acid signalling on wheat microbiome are dependent on soil type

Overview

Determining whether plant microbiomes are influenced by host plant defence signalling pathways is important for at least two reasons. Firstly, these pathways can be induced by external stimuli and have the potential to provide a mechanism to alter the microbiome structure towards plant-beneficial interactions. This may help illustrate the role of plant-associated microbiomes in plant nutrition and plant defence upon biotic attacks. In this chapter, I continued to examine the effects of the activation of SA signalling pathways on wheat associated microbial communities. I used a real-time quantative PCR assay to verify if the exogenious treatment with SA activates the SA signalling pathway in wheat, and then used 16S rRNA gene amplicon sequencing to determine the composition and diversity of the wheat associated microbial communities.

Highlights

- Elevated SA signalling led to a marginally significant change in the composition of wheat rhizosphere microbial communities in a Solonetz but not in a Calcisol soil;
- In the Solonetz wheat rhizosphere, SA signalling was negatively associated with the abundance of *arch-amoA, nifH*, *amoA* and *nosZ* nitrogen cycling gene transcripts and the relative abundance of a *Candidatus Nitrososphaera* population.
- In the Solonetz wheat rhizosphere, SA signalling was also positively associated with the relative abundance of a *Lysobacter*-like population, close relatives of which are known biocontrol agents.

Effects of salicylic acid signalling on wheat microbiome are dependent on soil type

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Abstract

Plant salicylic acid (SA) signalling pathway regulates plant growth, development and also mediates plant defence against biotrophic pathogens. However, it is not known yet if SA signalling influences the monocotyledonous plant associated microbiome, e.g., the microbial composition and function in rhizosphere. In the present study, we tested the effect of the activation of SA signalling on the composition and function of wheat (*Triticum* spp.) rhizosphere microbial communities. Wheat was grown in two field soils (Solonetz and Calcisol) that have been used for continuous wheat cropping for many years. SA was exogenously applied to the shoots of 10 day-old's wheat seedlings and the rhizosphere soils were collected 72 h after SA treatment. High throughput phylogenetic marker gene sequencing (16S rRNA gene) was used to assess bacterial and archaeal communities after the SA signalling activation. The genes *ChitinaseA*, *nifH, arch-amoA*, *amoA*, *nosZ* and *narG* that are involved in key reactions of either carbon or nitrogen cycling were quantified to determine the potential changes in function of rhizosphere soil. The enhanced SA signalling marginally changed the composition of the rhizosphere microbial communities in Solonetz (P=0.093) but not in Calcisol (P=0.31) rhizosphere. In particular, SA signalling increased the abundance of a close relative to *Lysobacter*, which is reported to be involved in biocontrol. SA also triggered a significant decrease in the occurrence of Archaea member *Candidatus Nitrososphaera,* and *Sphingobacteria* but only in the Solonetz rhizosphere. SA treatment on an Archaea enriched soil confirmed the suppression of Archaea. Copy numbers of *arch-amoA, nifH*, *amoA* and *nosZ* were reduced in Solonetz rhizosphere by SA treatment, as revealed by quantitative PCR. Our findings suggest that SA signalling may alter the wheat rhizosphere microbiome and lead to a decrease in the abundance of archaeal and bacterial populations involved in nitrogen cycling in a soil type dependent manner.

Keywords

Archaea; carbon and nitrogen; plant defence; rhizosphere; salicylic acid; wheat

Abbreviations

amoA- ammoniamonooxygenase subunit A gene; *arch-amoA*- Archaea ammoniamonooxygenase subunit A gene; *narG*- nitrate reductase gene; *nifH*- nitrogenase gene; *nosZ*- nitrous oxide reductase gene; *SA*- salicylic acid

1. Introduction

Rhizosphere is a narrow soil zone surrounding plant roots, which acts as an interface for multiple interactions between soil microorganisms and plants (Philippot et al. 2013). A mounting number of studies have described the 'rhizosphere effect' as the increase in number and activity of soil microbes in this zone as a consequence of higher availability of root exudates compared with bulk soil (Philippot et al. 2013; Sørensen et al. 1997). These microorganisms can exert a significant influence on plant health and growth, being even considered as the plant second genome (Berendsen et al. 2012). These microbes from complex assemblages of different taxonomic groups, whose composition of microbial communities have been reported to play a key role in ecosystem functions (Reed et al. 2007; Strickland et al. 2009). Plant and soil factors which affect microbial composition include plant genotype (Berendsen et al. 2012; Patel et al. 2015), development stage (Edwards et al. 2015), physiological conditions, and soil edaphic properties such as pH, soil type and moisture (Berg and Smalla 2009). There is evidence to suggest that plant hormone signalling, e.g., salicylic acid (SA) and jamonic acid (JA) may also play a role in the interactions between plants and their associated microboimes (Carvalhais et al. 2013; Lebeis et al. 2015). Deciphering whether/how the activation of plant defence signalling influences the microbial community structures in the rhizosphere can link soil microbial communities with plant defence and growth, and thus shed light to a future more sustainable agriculture.

In *Arabidopsis thaliana*, it has been demonstrated that variations in JA and SA signalling affect the composition of root-associated bacterial communities. Carvalhais *et al*. (2013) revealed changes in the composition of rhizosphere bacterial communities of *Arabidopsis* upon activated JA signalling generated by exogenous methyl jasmonate treatment. It was hypothesized that the abundance of defence-related bacterial populations including *Bacillus* and *Lysinibacillus* while potential growth promoting microorganisms of *Pseudomonas* spp. decreased in abundance. In addition, the *Arabidopsis* mutants *myc2* and *med25* which are impaired in the JA signalling released distinct root exudate profiles from the wild-type (Carvalhais et al. 2015). The discrimination

between the *Arabidopsis* genotypes attributed to the root exudate profiles and significantly correlated with differences in the microbial community composition in the rhizosphere (Carvalhais et al. 2015). These two studies provide evidence to suggest that plant JA signalling can sculpt microbial communities in rhizosphere.

SA mediates another signalling defence pathway that is typically antagonistic to JA (Pieterse et al. 2009). This hormone plays a pivotal role in plant defence against biotrophic pathogens. A recent study by Lebeis et al. (2015) found that isogenic *Arabidopsis* mutants with altered SA signalling harboured a distinct root endophytic bacterial community compared with the wild-type. This suggests that *Arabidopsis* requires the SA signalling pathway to modulate the colonisation of specific bacterial families on roots and drives the selection of microbial communities to sculpt root microbiome. However, no major changes in rhizosphere bacterial community diversity were observed in this study. Yet, there is no information whether the soil type and the plant species plays a role on the responses of the rhizosphere microbiome to the activation of the SA signalling pathway.

The soil microbiome mediates key ecological processes such as nitrogen (N) fixation, ammonification, nitrification and denitrification, therefore influencing soil available N in agricultural and natural systems (Galloway et al. 2008). Previously, ammonia-oxidizing bacteria (AOB) have been recognised as the only microbes responsible for ammonia oxidation, which is the rate-limiting step of nitrification (Kowalchuk and Stephen 2001). Recently, metagenomic analysis and the isolation of ammonia-oxidizing archaea (AOA) revealed the presence of putative ammonia monooxygenase subunits in the ubiquitous Archaea of the Thaumarchaeota phylum (Könneke et al. 2005; Leininger et al. 2006). These findings indicate that AOA is also involved in nitrification. It has been demonstrated that determination of the abundance of N cycling genes can better predict soil N-cycling because they reveal the current soil process, which can be superior to other physico-chemical and biological parameters such as pH, water content, and N and ammonium content (Petersen et al. 2012). Despite the importance of various microbes to N-cycling, the effect of plant defence signalling pathways on the abundance of functional genes from the rhizosphere microbiome is still unclear.

In the present study, we tested the hypothesis that the activation of SA signalling impacts microbial community composition and N cycling in wheat rhizosphere. We used phylogenetic high throughput marker gene sequencing (16S rRNA gene) to assess changes in the composition, richness and evenness of microbial communities in wheat rhizosphere. We also quantified the

abundance of *ChitinaseA*, which is involved in C cycling; five genes associated with N-cycling, namely the N fixation gene *nifH*, the amonification genes *amoA* (ammonia-oxidizing bacteria, AOB) and *arch-amoA* (ammonia-oxidizing Archaea, AOA), the nitrate reductase gene (*narG*) and nitrous oxide reductase gene (*nosZ*). The abundances of these genes were determined as a proxy of the N-cycling potential of the microbial communities in wheat rhizosphere.

2. Results

2.1 Activation of the SA signalling pathway in wheat seedlings

The transcriptional levels of *WCI2* and *WCI3*, which have previously been shown to be associated with activation of the wheat SA signalling pathway (Sardesai et al. 2005), were quantified in shoot tissues 72 hours after SA application using real-time PCR (Fig. 1). For plants grown in the Solonetz soil, SA application led to significant 52.4-fold and 96.7-fold increases in the abundance of *WCI2* and *WCI3* gene transcripts relative to the controls, respectively (Fig. 1). In the Calcisol soil, SA application led to significant 9.0-fold and 86.8-fold increases in the abundance of *WCI2* and *WCI3* gene transcripts relative to the controls, respectively (Fig. 1). These results indicate that the SA treatment was successful in activating the SA signalling pathway.

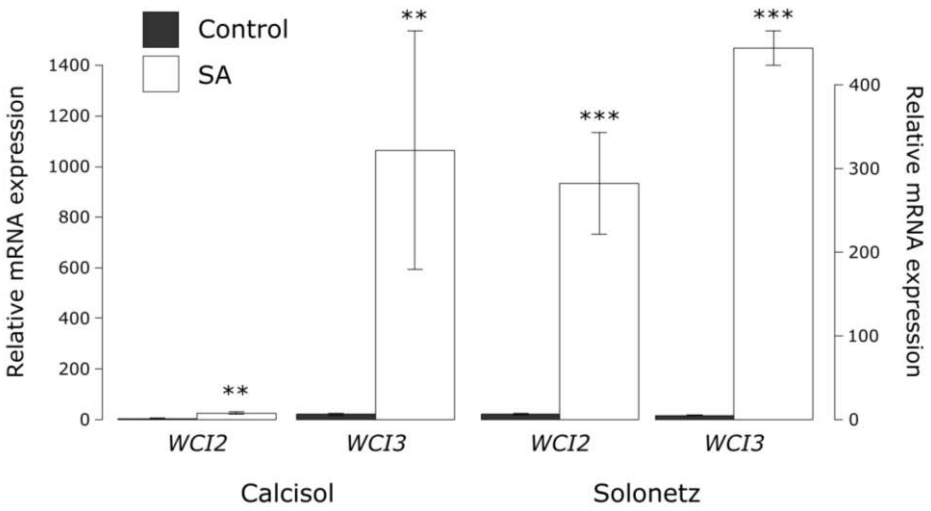

Fig. 1 The effect of salicylic acid (SA) application on the transcription of genes associated with the SA signalling pathway in 10-day-old wheat seedlings grown in different soil types. Asterisks indicate significant differences between control and SA treated plants ($P < 0.01^{**}$, $P < 0.001^{***}$, ANOVA). Error bars represent standard errors of the means (n = 3).

2.2 Rhizosphere bacterial and archaeal communities were influenced by SA treatment in Solonetz soil but not in Calcisol soil

In the Solonetz, 19 of the most dominant OTUs (present at higher than 1% relative abundance) in the rhizosphere were identified as bacteria, and one dominant archaeal population was affiliated to the genus *Nitrososphaera* (Fig.S2a). In Solonetz the bulk soil, 14 and 6 OTUs were as affiliated to bacteria and archaea, respectively (Fig.S2b). In the Calcisol, 31 and 2 dominant OTUs in the rhizosphere and bulk soil were affiliated to bacteria and archaea, respectively (Fig.S2c). The summarised OTUs for archaea and each bacterial phylum in the rhizosphere are shown in Fig.2. Interestingly, the activation of the SA signalling defence pathway led to changes in abundance of several bacterial phyla and archaea in the rhizosphere of the Solonetz but not of the Calcisol (Fig.2a,b). Overall, the exogenous SA treatment on plants cultivated in Solonetz caused a marginally significant change in bacterial and archaeal OTU relative abundances (P=0.093, RDA) (Fig.2a). Meanwhile, in the Calcisol enhanced SA signalling did not cause major changes in bacterial and archaeal composition, either in the rhizosphere (P=0.41, RDA) or bulk soils (P=0.305, RDA) (Figs.2 and 3, Fig.S2c). At phylum level, in the rhizosphere of plants grown in Solonetz, Proteobacteria were significantly increased (P=0.007, two-talied t test) while archaea (P=0.038), Gemmatimonadetes (P=0.0005), Armatimonadetes (P=0.01) and Cyanobacteria (P=0.039) were decreased in abundance by SA treatment (Fig. 2).

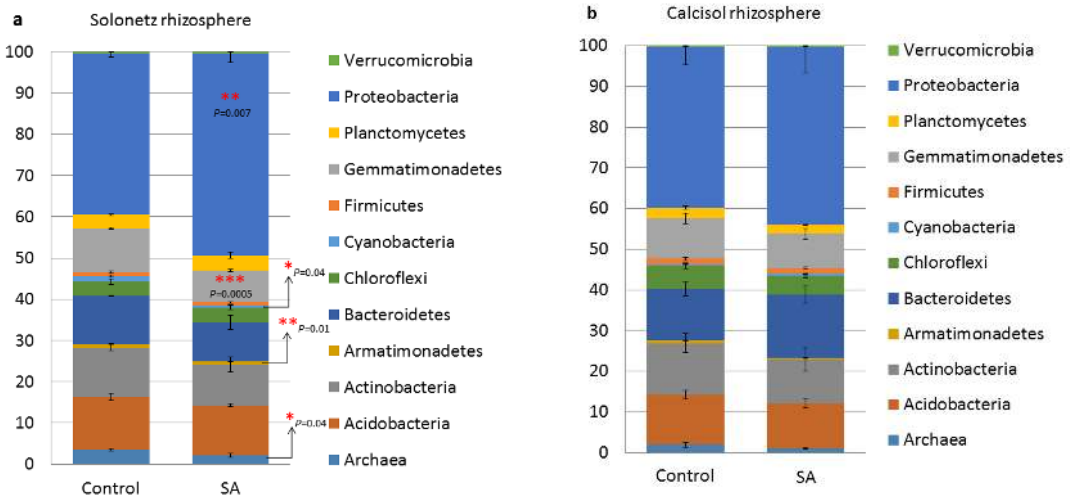

Fig. 2 Relative abundances of bacterial and archaeal groups in wheat rhizosphere at phylum level (color-coded) based on taxonomical classification of 16S rRNA gene amplicons with Solonetz soil in figure a and Calcisol soil in figure b. The star(s) in figure a represent significant differences

between control and SA treatments (n=3, two-tailed student *t* test, $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)).

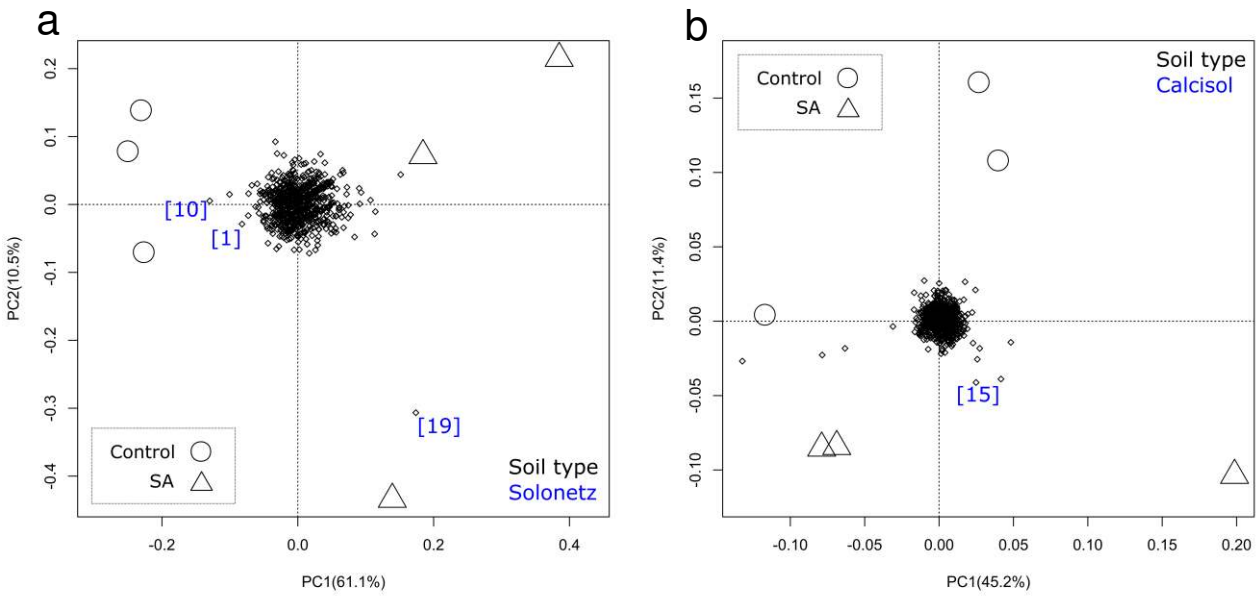

Fig. 3 Principal Component Analysis (PCA) graphs showing the distribution of bacterial and archaeal communities based on the relative abundance of Operational Taxonomic Units (OTUs) in rhizosphere of SA-treated wheat cultivated in a Solonetz (a) and a Calcisol soil (b). Small circles represent individual OTU.

Six bacterial or archaeal OTUs were changed in abundance by the activation of SA signalling in the rhizosphere of the wheat grown in Solonetz as follows: c_Proteobacteria [14] (*P*=0.003, 1.89 fold) and g_*Lysobacter* [20] (*P*=0.016, 1.76 fold) increased in relative abundance (Fig.4, Fig.S2a). Although it was not statistically significant, g_*Pseudomoas* [19] showed a trend of increase upon SA treatment (Fig.S2a). Alternatively, g_*Candidatus Nitrososphaera* [1] (*P*=0.03, 0.52 fold), s_*Streptomyces griseoaurantiacus* [8] (*P*= 0.05; 0.44 fold), o_Spingobacteria [10] (*P*=0.02, 0.46 fold) and f_*Gemmatimonadaceae* [13] (*P*=0.02, 0.51 fold) decreased in relative abundance. To confirm the observed decrease in archaea, we also tested the effect of SA signalling on a Solonetz soil which has a naturally high relative abundance of archaea compared to most soils (12.9%) using 16S rRNA gene amplicon high throughput sequencing. Consistently, a marginally significant decrease in total Archaea (*P*=0.054, 0.58 fold) and a significant decrease in g_*Candidatus Nitrososphaera* (0.49 fold, *P*=0.037) was observed 48 h after exogenous SA treatment (Fig.5).

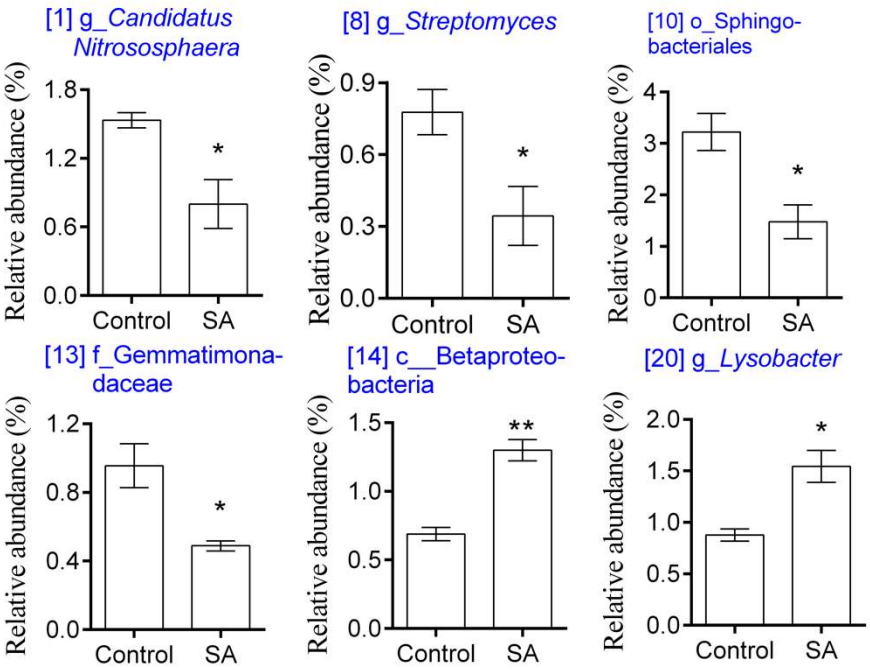

Fig. 4 The OTUs (>1%) that were affected in relative abundance in Solonetz wheat rhizosphere. The difference between treatments for each OTU is reflected by the asterisk(s) above the columns ($P < 0.05$ (*), $P < 0.01$ (**)). Numbers of [1], [8], [10], [13], [14] and [20] in this figure correspond to the same OTUs as those in Fig.S2a.

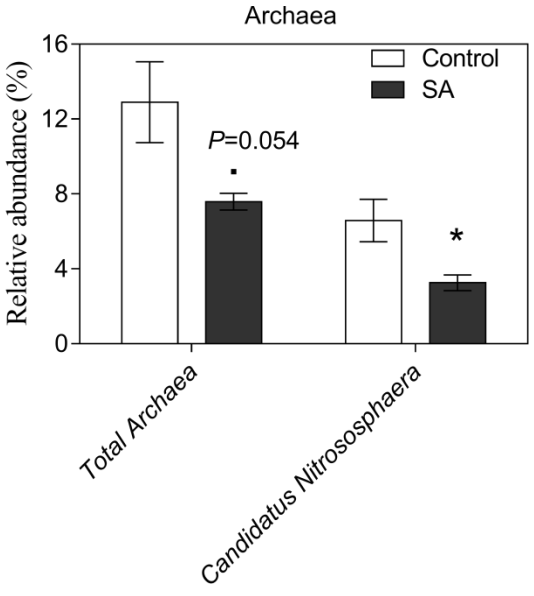

Fig. 5 Summarisation of proportion of archaeal groups in total archaeal and bacterial communities. In terms of the experiment, the soil used for wheat cultivation was a Solonetz which has high abundance of archaeal components (12.90%) in the bacterial and archaeal communities. The star above the arrow bar represents significant difference between control and SA treatment (n=3, two-tailed student *t* test, $P<0.1$ (·); $P < 0.05$ (*)).

In the Calcisol the composition of bacterial and archaeal communities of rhizosphere and non-rhizosphere were clearly distinct (P<0.005, RDA). In the Calcisol, rhizosphere microbial communities were less diverse than in non-rhizosphere soils with reduced representations of Acidobacteria, Archaea and Chloroflexi and enrichment of Bacteroidetes and Proteobacteria (ANOVA, P<0.005, Fig.S2c). The profiling of the bacterial and archaeal communities in the Solonetz rhizosphere and non-rhizosphere soil has been done in different experiments. Therefore, we decided not to evaluate the rhizosphere effect given the possible variation in the source communities present in the non-rhizosphere soil.

2.3 SA effects on the abundance of ChitinaseA and five N cycling genes

In both Solonetz and Calcisol rhizosphere soils, the relative copy numbers of *ChitinaseA* and N cycling genes were as follows: *nifH* > *ChitinaseA* > *arch-amoA* > *amoA* > *nosZ* > *narG* (Table1). Except for the *ChitinaseA*, Solonetz had higher gene copy numbers of *arch-amoA*, *amoA*, *nifH* and *narG* than in Calcisol. The copy numbers of *arch-amoA* (0.68 fold, P=0.007), *nifH* (0.63, P=0.009), *amoA* (0.32, P=0.003) and *nosZ* (0.62, P=0.03) were significantly decreased while the copy numbers of *ChitinaseA* (P=0.08) were marginally decreased by the activation of SA treatment in the Solonetz wheat rhizosphere (Table 1). In Solonetz non-rhizosphere and Calcisol rhizosphere soil, SA treatment did not cause decreases in gene abundances (Table1, Table S5).

Table 1 Abundance of bacterial and archaeal functional genes *arch-amoA*, *ChitinaseA*, *amoA*, *nifH*, *narG* and *nosZ* in wheat rhizosphere 72 h after the exogenous SA treatment. '-' represents that gene expressions were not detected (either qPCRs Ct>40 or amplifications were not detected). Asterisks values indicate differences between treatments (two-tailed Student *t* test, P <0.1 (.), P < 0.05 (*), and P < 0.01 (**). P = 0.08 for *ChitinaseA* was detected between treatments in Solonetz wheat rhizosphere. Standard deviations of the mean are shown (n=3).

	Solonetz		Calcisol	
	Control	**SA**	**Control**	**SA**
arch-amoA	665.9±60.0	348.0±23.4**	17.9±4.1	7.38±2.49
ChitinaseA	3453.9±325.8	2338.9±334.0.	4434.7±347.3	2579.8±808.7
amoA	86.0±5.5	33.0±6.1**	3.78±0.46	3.05±0.47
nifH	19370.0±394.0	14461.9±1003.4**	10536.0±1198.8	10106.9±1511.0
narG	3.7±0.9	1.6±0.27	-	-
nosZ	14.5±0.29	10.3±1.2*	11.4±2.2	10.4±0.8

3. Discussion

In this study, we investigated how an artificially elevated SA signalling affects rhizosphere microbiome in wheat. Our results support the hypothesis that depending on the soil type the activation of SA signalling alters the bacterial and archaeal communities and the potential microbial function in wheat rhizosphere. Our key findings were that the activation of the SA signalling pathway reduced the abundance of archaea and also copy numbers of four genes involved in N cycling (*arch-amoA*, *nifH*, *amoA* and *nosZ*) in the rhizosphere of wheat grown in a Solonetz, but not in a Calsisol.

3.1 Activation of the SA signalling pathway

Gene expression of *WCI2* and *WCI3* in the shoots of wheat seedlings grown in either Solonetz or Calcisol were significantly induced 72 h after SA treatment. *WCI* genes have been associated to systemic acquired resistance (SAR) and a specific set of *WCI* genes have been previously induced by the SA analog benzothiadiazole (BTH) (Görlach et al. 1996). The induction of *WCI* genes has been involved in an increased resistance of wheat to powdery mildew infection through affecting multiple steps of the pathogen development (Görlach et al. 1996). In summary, the induction of *WCI2* and *WCI3* provides enough evidence to suggest that SA signalling defence pathway was activated by the SA treatment on wheat seedlings growing in both Solonetz and Calcisol.

3.2 The effects of an elevated SA signalling on wheat rhizosphere microbial communities

SA is a pivotal mediator of SAR whose synthesis is enhanced by exogenous treatment with SA. The PCA based on the relative OTU abundances shows distinct spatial separation patterns of the samples corresponding to the different treatments in the Solonetz compared to the Calcisol. In the secondary axis of the PCA, there is a clear separation of SA treated samples from the control in the Solonetz, which is not the case for both primary and secondary axes of the PCA in the Calcisol (Figs. 3a, b). This suggests that the rhizosphere microbial communities responded to SA signalling differently in these two soil types (Fig.3). The fact that there were no major changes in OTU relative abundances in the SA-treated bulk soil indicate that the results observed were not a consequence of a direct effect of the SA treatment on the soil (Fig. S2b,c). In addition, we also collected Calcisol wheat rhizosphere soil 48 h after SA treatment, and did not observe any changes

in the soil microbial community composition or functional gene abundances (data not shown). These findings suggest that the microbial communities of Calcisol were more resilient to the SA treatment. Therefore, the responses of the plant rhizosphere microbial communities to an elevated SA signalling occurred in a soil type dependent manner.

The marginally significant changes in microbial community composition suggest that SA affects some taxonomic groups of bacteria at the genus level in the rhizosphere (P= 0.093, Solonetz; P=0.305, PERMANOVA). Several dominant OTUs whose relative abundance changed by SA treatment in Solonetz rhizosphere are affiliated to groups that have been reported to perform important ecological functions and potentially influence plant health and performance. For instance, Sphingobacteria which is a class belonging to the phylum Bacteroidetes decreased in relative abundance among the microbial communities in Solonetz rhizosphere. Sphingobacteria are known for their fermentative metabolism and the degradation of polysaccharides derived from plant material (Turnbaugh et al. 2011). An increase in decomposition of recalcitrant C compounds in a fertilized soil has been reported to correlate with an increased abundance in Bacteroidetes and Gemmatimonadetes (Nemergut et al. 2008). Moreover, Sphingobacteria are capable of producing sphingolipids that play a pivotal role in plant programmed cell death, cell recognition and signalling, and membrane subdomain formation during plant defence response (Berkey et al. 2012; Heung et al. 2006; Olsen and Jantzen, 2011). Therefore, the decrease in Sphingobacteria in wheat rhizosphere may also alter plant physiological conditions.

Archaea perform key biochemical reactions in agricultural and natural ecosystems such as ammonia oxidation and methanogenesis (Offre et al. 2013). To our knowledge, our study for the first time to reveal that abundance of the rhizosphere ammonia-oxidizing Archaea (AOA), in particular *Nitrososphaera gargensis* was suppressed by an elevated plant SA signalling. *Nitrososphaera gargensis* possesses ammonia monooxygenase and carries out oxidation of ammonia (NH_4^+) into nitrite (NO_2^-), which is an essential step in the N cycle. Despite the fact AOB and AOA have been detected in both Solonetz and Calcisol in the present study, AOA are dominate in both soils as more copy numbers of *arch-amoA* than *amoA* were quantified, which suggests that *Thaumarchaeota* may be significant contributors to ammonia oxidation in these two soils. Nitrification is a very relevant process in agricultural systems because it converts fertilisers in the form of ammonia to nitrate (NO_3^-), which is a more soluble form of N and therefore prone to leaching (Galloway et al. 2008). We also found that the abundance of Gemmatimonadetes was decreased in the Solonetz rhizosphere of SA-treated plants. The Gemmatimonadetes have been

found in a variety of arid soils, which suggest that they are well adapted to low soil moisture (DeBruyn et al. 2011).

Members of the genus *Lysobacter*, belong to the family *Xanthomonadaceae* within the Gammaproteobacteria, have been widely considered as PGPR for their biocontrol properties through activating defence mechanisms of induced resistance and production of a variety of novel antibiotics such as katanosins (Islam 2011). A larger number of *Lysobacter* spp. have been implicated in disease suppression against potato common scab (Rosenzweig et al. 2011), in suppression of damping-off disease in host plants, in biological control of wheat *Fusarium* head blight blight (Jochum et al. 2006) and in control of the *Bipolaris sorokiniana* which causes leaf spot of tall fescue (Zhang and Yuen 1999). The antifungal properties of *Lysobacter* seem to be so general that there are reports of its involvement in the protection of the red-backed salamander against the fungal pathogen that causes chytridiomycosis (Brucker et al. 2008). The increase in *Lysobacter* may indicate that defence-related bacteria were recruited to the wheat rhizosphere by the activation of SA signalling. The decrease in archaea, Bacteroidetes and Gemmatimonadetes may indicate a decrease in the decomposition ability of the wheat rhizosphere upon enhanced SA signalling in the Solonetz. Except for Streptomycetes, Cyanobacteria, Gemmatimonadetes, Spingobacteria, Proteobacteria (and therefore *Lysobacter*, *Pseudomonas*) and *Candidatus Nitrososphaera* are all gram negative (Jung et al. 2014). Therefore, we speculate that Gram negative microbes in rhizosphere may be more influenced by the enhanced SA signalling in wheat. In agreement with our findings, SA signalling in *Arabidopsis* has been reported to modulate rhizosphere bacteria community composition (Lebeis et al. 2015).

3.3 Effect of SA signalling on the abundance of genes involved in carbon (C) and nitrogen cycling in wheat rhizosphere

Free-living (e.g. *Azotobacter* spp.) and symbiotic (e.g. *Rhizobium* spp.) N-fixing bacteria and some archaea have the nitrogenase enzyme complex, which convert gaseous N_2 into ammonium (NH_4^+), providing the main source of N in terrestrial ecosystems (Galloway et al. 2004). Nitrifying microbes oxidize NH_4^+ into nitrate (NO_3^-) having nitrite (NO_2^-) as an intermediate. Denitrifying microbes reduce NO_3^- into nitric oxide (NO), nitrous oxide (N_2O) and eventually to N_2, returning N to the atmosphere and therefore completing the N cycle (Galloway et al. 2004). In the present study, we observed significant decreases in abundances of *arch-amoA*, *nifH*, *amoA* and *nosZ* in Solonetz rhizosphere after SA treatments. These results are consistent with the decrease in *archaea* and the

bacterial phyla *Cyanobacteria*, *Gemmatimonadetes* and *Sphingobacteria*. Particularly, Sphingobacteria have been reported to be involved in biogeochemical transformations involving N cycling (Bier et al. 2015).

A significantly lower nitrification potential compared with the unsown soil has been found in *faba* bean crop. Soil type and plant species affected the community composition of AOB, AOA, and N_2-fixers (*nifH*). *AOA* and *nifH* community composition have been reported to be sensitive to environmental conditions such as crop species, cropping system and soil type (Wang et al. 2012a). The *arch-amoA* was the main oxidizer in ammonium oxidation with gene copy numbers more than one hundred times greater than those of the AOB-*amoA* in both soil types (Wang et al. 2012a). Environmental parameters such as pH, nutrients and oxygen levels determined the phylotypes and the diversity of the ammonia oxidizers in soils (Wang et al. 2012b).

3.4 Linking soil type and SA signalling

The different responses of the wheat rhizosphere microbial communities to SA signalling may have been caused by the distinct soil edaphic properties and nutrient contents in Solonetz and Calsisol. For instance, the Solonetz has more nutrients than the Calcisol regarding total C (+2.2 fold), organic C (+2.2 fold), total N (+3.5 fold), total microbial activity (as indicated by fluorescein diacetate, +2.2 fold) and microbial biomass C (+2.6 folds) but has a similar metabolic activity (Microresp^{TM}-CLPP method, +0.96 fold) (Table S1; Liu et al., 2016 b,c). In addition, Solonetz has less clay content than Calcisol. The Solonetz used in the present study is one of the most agronomically productive soils in the Australian Northern Grains Region (Liu et al., 2016 b,c,d; Bell et al., 2006). The abovementioned differences between the two soils indicate that the rhizosphere microbial communities in high nutrient soils may be more likely respond when plants activate SA-mediated signalling pathways. This may also suggest that plants growing in fertile soils possibly respond to disease attacks by altering rhizosphere microbial communities.

4 Conclusions

Our finding revealed that wheat rhizosphere archaeal and bacterial communities respond to an elevated signalling pathway differentially depending on the soil type (Fig.6). However, the underpinning principles of how these soils influence microbial interactions with plants activating a defence signalling pathway still warrants further investigation. Plants are sessile; therefore, during

evolution they have developed a cost-efficient manner to tackle unfavourable conditions. We hypothesized that plants recruit *Lysobacter* spp. to the rhizosphere during the activation of the SA signalling pathway, possibly to act as a biocontrol agent. Moreover, several microbial taxa that are reported to be involved in N cycling, degradation and metabolism of organic matters were suppressed in the rhizosphere upon SA signalling, especially the archaeal groups. Interestingly, Sphingobacteria which have been reported to produce sphingolipids were reduced in abundance in the rhizosphere by enhanced SA signalling pathway. Determining the content of Spingolipids in rhizosphere and how they interact with plant defence signalling warrants further investigation.

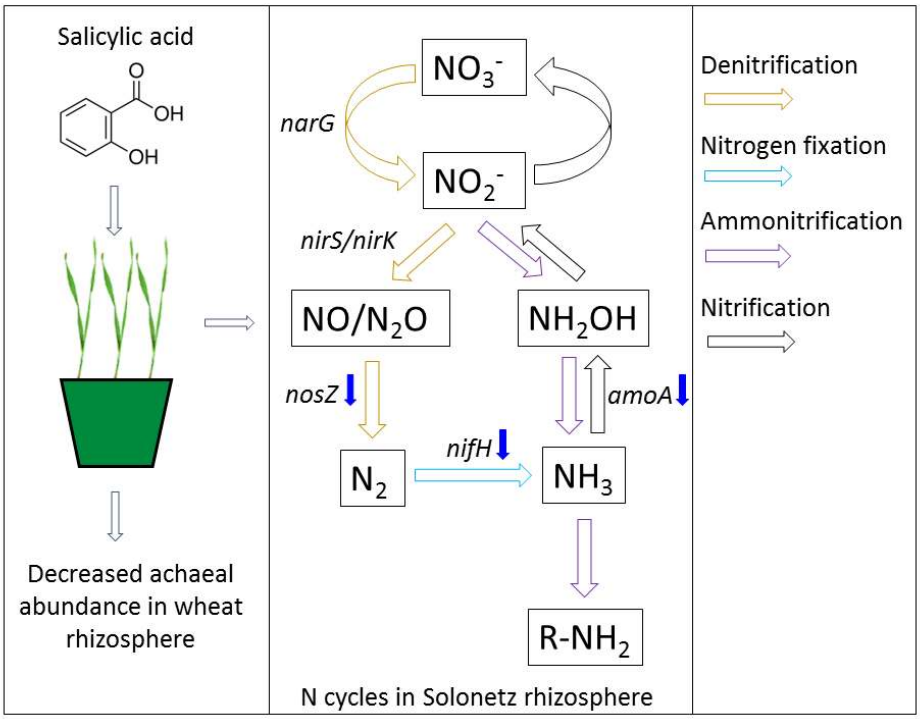

Fig. 6 Schematic graph showing that several functional genes involved in key N cycling reactions in Solonetz wheat rhizosphere soil changed in abundance by SA treatments. Elevated SA signalling led to decreases in the abundance of *nifH*, *amoA* and *nosZ*, and the relative abundance of Archaea among microbial communities. Furthermore, these SA induced changes in wheat rhizosphere were detected in a soil-dependent manner (only detected in Solonetz but not Calcisol soil). The blue arrows denote decrease in copy numbers of N cycling genes.

5 Materials and Methods

5.1 Soil description, experimental treatments and rhizosphere soil sampling

Triticum aestivum variety 'Crusader' plants were cultivated in two different soils, one collected from Condamine (26.90°S, 149.64°E) and the other from Moonie (27.79°S, 150.20°E) in the Australian Northern Grains Region in August, 2013. These two agricultural soils have been previously studied (Liu et al, 2016 b,c), and the physicochemical composition of organic C and N, phosphorus, potassium, zinc, ferrous, manganese, copper and soil pH is listed in Table S1. The soil collected from Condamine has about 2.5 times more nutrients than the collected Moonie soil (Table S1). These two soils were classified as a brown Solonetz and a grey Calcisol, respectively (IUSS, Working Group WRB, 2007). The soil collected from Condamine and Moonie has a 19- and 7- year conservation farming history, respectively. Wheat was the last crop for both the Solonetz and Calcisol before soil collection. These soils were collected from the surface profile (0-10 cm), which were also sieved (<5 mm porosity) and homogenized to ensure homogeneity before planting.

A schematic graph showing the experimental design and sampling strategy is shown in Fig.S1. Plants were cultivated in two trays and grown in a controlled environment chamber (Percival Scientific, Boone, IA, USA) at 24°C with a light intensity of 150 mmol m^{-2} s^{-1}. Three biological replicates were used per treatment. The SA solution (5 mM) used for spraying was prepared by dissolving 13.81 mg SA in 1 mL absolute ethanol, which was then further dissolved in 20 mL of milli-Q water. The SA solution was sprayed on 10-day wheat seedlings (2 leaf-stage) until droplets visibly covered the shoot surface (sprayed volume of approx. 0.67 mL per plant). As control, 5% ethanol (without SA) was sprayed on the 10 day-wheat seedlings. Parallelly, two trays of soils (without wheat plantation) were mock and SA treated using the abovementioned method (Fig.S1). Seventy-two hours after treatments, the rhizosphere soil was collected and preserved in phosphate buffer at -80°C until further use. Wheat shoots and bulk soil were also stored at -80°C for confirming the activation of SA signalling pathway and the direct effect of SA on soil microbiome, respectively.

5.2 Extraction of total wheat RNA, soil genomic DNA and qRT-PCR implementation

Plant total RNA isolation and qRT-PCR were performed as previously reported (Liu et al. 2016 a). Briefly, the harvested shoot samples were ground in liquid nitrogen with a mortar and pestle, and plant RNA was isolated using the SV Total RNA Isolation Kit (Promega). The cDNA was synthesized by reverse transcription using Superscript III kit (Life Technologies) from 1.5 µg of total RNA in a 20 µL reaction. The relative quantification of mRNA expression was performed using SYBR Green RT-PCR mixtures on a ViiA™ 7 Real-Time PCR system (Applied Biosystems,

USA). Wheat chemical induced gene 2 (*WCI2*) and wheat chemical induced gene 3 (*WCI3*) were selected to verify the activation of the SA signalling pathway. qRT-PCR data analysis was then performed via ViiA 7 RUO Software (Applied Biosystems) using the *18S rRNA* gene as an endogenous reference for normalization. The primer sequences are listed in Table S2. cDNA used for quantifying the *18S rRNA* were diluted 500 times because of the high expression levels of this particular gene.

Soil DNA was extracted from 0.25~0.40 g soil per sample using the Powersoil Kit (MOBIO Laboratories, CA). DNA concentration was determined using a Qubit^{TM} fluorometer with Quant-iT dsDNA BR Assay Kits (Invitrogen) and then normalised to 2.5 ng μL^{-1}.

5.3 PCR for 16S amplicon pyrosequencing

Eubacterial and archaeal 16S rRNA genes were amplified by PCR in a 25 µL reaction using the universal ribosomal 16S primers of 926F (5'-AAA CTY AAA KGA ATT GRC GG-3') conjugated with B adapater (3'-CCT ATC CCC TGT GTG CCT TGG CAG TC-5'), and 1392R (3'-ACG GGC GGT GWG TRC-5') conjugated with A adapter (3'-CCA TCT CAT CCC TGC GTG TCT CCG AC-5') and barcodes. This primer pair preferentially amplifies archaeal and bacterial DNA and prevents amplification of host (plant) eukaryotic DNA. PCR reactions included 5.0 µL 5×Phire buffer, 1.25 µL 10 µM dNTP (Invitrogen), 1.25 µL 10 µM Pyro_1392 R, 1.25 µL 10 µM Pyro_926 F, 0.5 µL phire® hot start II and molecular biology grade water to 25 µM. Cycling conditions used for amplification started with an initial denaturation at 98 °C for 5 min, followed by 35 cycles of 98 °C 15 s, 55 °C for 15s and with a 72 °C for 45s; with a final elongation at 72 °C for 7 min. For each sample, three amplifications were carried out to minimize PCR bias. A no template control was used to check for contamination. After size examination on a 1.5% agarose gel, the PCR products of the same sample were combined and purified with Wizard® SV Gel and PCR Clean-Up System (Promega). All purified amplicons were adjusted to 50 ng μL^{-1}, pooled, and further purified using the same kit. The purified PCR products were sent to Macrogen (Korea) for 454 sequencing (Roche).

5.4 qPCR for profiling Chitinase groupA and N-cycling genes

The primers used for measuring copy numbers of the *Chitinase groupA* and genes involved in N cycling (*arch-amoA*, *amoA*, *nifH*, *nosZ* and *narG*) are listed in table S3. All qPCR reactions

contained 1.5 µL 0.3 µM of each forward and reverse primer, 5 µL 2×Faststart SYBR green mix (Roche Diagnostics Ltd), 1 µL DNA template (2.5 ng) and 2.5 µL DNase free water. Cycling conditions included an initial step at 98°C for 10 minutes; followed by 45 cycles of 98°C for 30 s, annealing for 45s, and elongation at 72 °C for 45 s. Dissociation curve were generated for each gene by adding the cycle 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s at reduced ramping rate of 0.2 °C/s to check for unspecific amplification. Data were analysed using Light Cycler® 96 software. The optimal annealing temperature for each primer pair was tested by using a temperature gradient. The optimised annealing temperatures were higher than what had been reported previously (Table S3). The specificity of qPCR amplification was firstly confirmed by a single melting peak, and secondly by a single band on a 1.5% agarose gel (Fig.S3). Bands were excised from the gel and then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Purified PCR products were sent to the Australian Genome Research Facility Ltd (AGRF) for Sanger sequencing. Dendograms showing distance-based clusterings generated by pairwise alignments with the query sequence in BLAST (https://www.ncbi.nlm.nih.gov/Web/Newsltr/V15N1/blastlab.html) are shown in Fig.S4A-F. Ten-fold sequential dilutions of purified amplification products were used to generate standard curves with the abovementioned conditions. The following standard curve equations for each gene were obtained: *ChitinaseA*: y=-1.0196x+35.043 (R^2=0.9859), *amoA*: y=-1.2275x+33.518 (R^2=0.9999), *arch-amoA*: y=-0.9814x+33.736 (R^2=0.9969), *nifH*: y=-1.6161x+47.827 (R^2=0.9948), *narG*: y=-1.8342x+36.707 (R^2=0.9900), and *nosZ*: y=-1.4453x+28.723 (R^2=0.9725). The gene copy numbers in the rhizosphere and bulk soil were quantified by comparing the Ct values gained by qPCR against the corresponding standard curve.

5.5 Bioinformatics for 16S pyrosequencing analysis and statistical analysis

Raw sequencing data from the 16S rRNA pyrosequencing were processed as described previously. Primer sequences were removed from each fastq file using the QIIME v1.9.1 script multiple_extract_barcodes.py. The header line of each sequence was then modified to contain a sample ID using a custom bash script and each sequence was quality filtered and dereplicated using the QIIME script multiple_split_libraries.py with the homopolymer filter deactivated (Caporaso et al. 2010). The forward reads from each sample were concatenated into a single file and checked for chimeras against the October 2013 release of the GreenGenes database using UCHIME ver. 3.0.617 (Edgar et al. 2011). Homopolymer errors were corrected using Acacia (Bragg et al. 2012). Sequences were then subjected to the following procedures using QIIME: 1) sequences were clustered at 97% similarity using UCLUST, 2) a representative sequence were randomly selected,

and GreenGenes taxonomy was assigned to the cluster representatives using BLAST as previously described, and 3) tables of an OTU list and their taxonomic assignments in each sample were generated. The number of reads was rarefied to 1,900 sequences per sample by re-sampling the OTU table. The mean number of observed (Sobs) and predicted (Chao1) Operational Taxonomic Units (OTUs) and Simpson's diversity index values were calculated using QIIME.

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Author contributions

H. L., P. M. S., L. C. C and P.G.D. conceived and designed the study. H. L. and L. C. C. performed experiments. P.G.D. and H. L. analysed the data and wrote the manuscript.

Competing financial interests

The authors declare no conflict of interest.

Supplementary information

Supplementary information is associated with the online version of this paper.

Data accessibility

The 16S rRNA amplicon sequences associated with this study have been deposited in the NCBI SRA under accession: PRJNA356991.

References

Berendsen RL, Pieterse CM, Bakker PA. (2012). The rhizosphere microbiome and plant health. Trends Plant Sci 17: 478-486.

Berg G, Smalla K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol 68: 1-13.

Berkey R, Bendigeri D, Xiao S. (2012). Sphingolipids and plant defense/disease: the "death"

connection and beyond. Front Plant Sci 3: 68.

Bier RL, Voss KA, Bernhardt ES. (2015). Bacterial community responses to a gradient of alkaline mountaintop mine drainage in Central Appalachian streams. ISME J 9: 1378-1390

Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW. (2012). Fast, accurate error-correction of amplicon pyrosequences using Acacia. Nat Meth 9:425-426.

Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KPC. (2008). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander plethodon cinereus. J Chem Ecol 34: 39-43.

Caporaso JG et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7:335-336.

Carvalhais LC, Dennis PG, Badri DV, Tyson GW, Vivanco JM, Schenk PM. (2013). Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. PLoS One 8: e56457.

Carvalhais LC, Dennis PG, Badri DV, Kidd BN, Vivanco JM, Schenk PM. (2015). Linking jasmonic acid signaling, root exudates, and rhizosphere microbiomes. Mol Plant Microbe In 28: 1049-1058.

DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M. (2011). Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. Appl Environ Microbiol 77: 6295-6300.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. (2011). UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-2200.

Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. P Nat Acad Sci 112: E911-E920.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland C, Green P, Holland E. (2004). Nitrogen cycles: past, present, and future. Biogeochemistry 70: 153-226.

Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320: 889-892.

Görlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T,

Ward E, Kessmann H. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8: 629-643.

Heung L, Luberto C, Del Poeta M. (2006). Role of sphingolipids in microbial pathogenesis. Infect Immun 74(1): 28-39.

Islam MT (2011) Potentials for biological control of plant diseases by *Lysobacter* spp., with special reference to strain SB-K88. Bacteria in agrobiology: Plant growth responses. Springer.

IUSS Working Group WRB. (2007). 'World reference base for soil resources 2006' World Soil Resources Report No. 103. ftp://ftp.fao.org/docrep/fao/009/a0510e/a0510e00.pdf Accessed on 13th December, 2016.

Jochum C, Osborne L, Yuen G. (2006). Fusarium head blight biological control with *Lysobacter enzymogenes* strain C3. Bio Control 39: 336-344.

Jung MY, Park SJ, Kim SJ, Kim JG, Damsté JSS, Jeon CO, Rhee SK. (2014). A mesophilic, autotrophic, ammonia-oxidizing archaeon of thaumarchaeal group I. 1a cultivated from a deep oligotrophic soil horizon. Appl Environ Microbiol 80: 3645-3655.

Könneke M, Bernhard AE, José R, Walker CB, Waterbury JB, Stahl DA. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437: 543-546.

Kowalchuk GA, Stephen JR. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu Rev Microbiol 55: 485-529.

Lebeis SL, Paredes SH, Lundberg DS, Breakfield N, Gehring J, McDonald M, Malfatti S, Glavina del Rio T, Jones CD, Tringe SG, Dangl JL. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science 349: 860-864.

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442: 806-809.

Liu H, Carvalhais LC, Kazan K, Schenk PM. (2016a). Development of marker genes for jasmonic acid signaling in shoots and roots of wheat. Plant Signal Behav 11: e1176654.

Liu H, Carvalhais LC, Rincon-Florez V, Crawford M, Dang YP, Dennis PG, Schenk PM. (2016b). One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol. Soil Till Res 158: 91-99.

Liu H, Carvalhais LC, Crawford M, Dang YP, Dennis PG, Schenk PM. (2016c). Strategic tillage

increased the relative abundance of Acidobacteria but did not impact on overall soil microbial properties of a 19-year no-till Solonetz. Biol Fertil Soils 52(7): 1021-1035.

Liu H, Crawford M, Carvalhais LC, Dang YP, Dennis PG, Schenk PM. (2016d). Strategic tillage on a Grey Vertosol after fifteen years of no-till management had no short-term impact on soil properties and agronomic productivity. Geoderma, 267:146-155.

Nemergut DR, Townsend AR, Sattin SR, Freeman KR, Fierer N, Neff JC, Bowman WD, Schadt CW, Weintraub MN, Schmidt SK. (2008). The effects of chronic nitrogen fertilization on alpine tundra soil microbial communities: implications for carbon and nitrogen cycling. Environ Microbiol 10: 3093-3105.

Offre P, Spang A, Schleper C. (2013). Archaea in biogeochemical cycles. Microbiology 67: 437.

Olsen I, Jantzen, E. (2011). Sphingolipids in bacteria and fungi. Anaerobe 7(2): 103-112.

Patel JS, Singh A, Singh HB, Sarma BK. (2015). Plant genotype, microbial recruitment and nutritional security. Front Plant Sci 6.

Petersen DG, Blazewicz SJ, Firestone M, Herman DJ, Turetsky M, Waldrop M. (2012). Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. Environ Microbiol 14: 993-1008.

Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nature Rev Microbiol 11(11):789-799.

Reed HE, Martiny JB. (2007). Testing the functional significance of microbial composition in natural communities. FEMS Microbiol Ecol 62(2): 161-170.

Rosenzweig N, Tiedje JM, Quensen JF, Meng Q, Hao JJ. (2011). Microbial communities associated with potato common scab-suppressive soil determined by pyrosequencing analyses. Plant Dis 96: 718-725.

Sardesai N, Subramanyam S, Nemacheck J, Williams CE. (2005). Modulation of defense-response gene expression in wheat during Hessian fly larval feeding. J Plant Interact 1(1): 39-50.

Strickland MS, Lauber C, Fierer N, Bradford MA. (2009). Testing the functional significance of microbial community composition. Ecology 90(2):441-451.

Sørensen J, Van Elsas JD, Trevors JT, Wellington EMH. (1997). The rhizosphere as a habitat for soil microorganisms. Modern Soil Microbiology (Van Elsas JD, Trevors JT, Wellington EMH eds), pp.21-45. Marcel Dekker, Inc., New York.

Turnbaugh PJ, Biomolecules SBD, Roscoff F. (2011). Environmental and gut *bacteroidetes*: the

food connection. Front Microbiol 2:93.

Wang Y, Marschner P, Zhang F. (2012a). Phosphorus pools and other soil properties in the rhizosphere of wheat and legumes growing in three soils in monoculture or as a mixture of wheat and legume. Plant Soil 354: 283-298.

Wang Y, Zhang F, Marschner P. (2012b). Soil pH is the main factor influencing growth and rhizosphere properties of wheat following different pre-crops. Plant Soil 360: 271-286.

Zhang Z, Yuen G. (1999). Biological control of Bipolaris sorokiniana on tall fescue by *Stenotrophomonas maltophilia* strain C3. Phytopathology 89: 817-822.

Supplementary Information

Effects of salicylic acid signalling on wheat microbiome are dependent on soil type

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Contents:

Table S1 Physicochemical characteristics of Solonetz and Calcisol soils.

Soil parameters	Solonetz	Calcisol
pH	7.4	7.8
Organic carbon content	1.28	0.59
Total carbon content	1.36	0.62
Total nitrogen	0.18	0.051
Fluorescein diacetate hydrolysis (fluorescein μg mL^{-1} g^{-1} soil h^{-1})	2.31	1.09
Microbial biomass carbon (mg C g dry $soil^{-1}$)	0.49	0.19
Total Al concentration (ppm)	30590	16380
Total Ca concentration (ppm)	1781	1162
Total Cu concentration (ppm)	17	3.7
Total Fe concentration (ppm)	21013	9455
Total K concentration (ppm)	1666	1010
Total Mg concentration (ppm)	154	269
Total Mn concentration (ppm)	1936	251
Total Na concentration (ppm)	933	253
Total P concentration (ppm)	278	86
Total S concentration (ppm)	355	1594
Total Zn concentration (ppm)	46	10
Clay (%)	25	31.2
Silt (%)	14	10.1
Sand (%)	61	58.7

Table S2 Primer sequences used for real-time PCR assays designed to confirm salicylic acid induced activation of SA signalling in wheat.

Accession	Gene	Forward	Reverse	Gene description	References
AF159369	*18S*	CAAAGCAAGCCTACGCTCT	ATACGAATGCCCCCGACT	*Haematococcus pluvialis* 18S ribosomal RNA gene	(Liu et al. 2016)
U32428	*WCI2*	TAGGAACTGGAACTTCACCGAGC	GGTAGTCCTTGATGTGCAGCGAC	wheat chemically induced (WCI) gene, lipoxygenase (fragment)	(Desmond, et al., 2006)
U32429	*WCI3*	AAAGTTGGTCTTGCCACTGACTG	TCGACAAAGCACTTCTGGATTTC	wheat chemically induced (*WCI*) gene, sulfur rich/thionin-like protein	(Desmond, et al., 2006)

Table S3 Primer sequences and annealing temperatures used for qPCR to determine the abundance of *ChitinaseA* and N cycling genes.

Genes	Forward sequence	Reverse sequence	Amplicon (bp)	Tm^A (°C)	Reference
ChitinaseA	CGTCGACATCGACTGGGARTDBCC	ACGCCGGTCCAGCCNCKNCCRTA	400	63	(Yergeau et al., 2007)
arch-amoA	TTCTTCTTTGTTGCCCAGTA	CTGAYTGGGCYTGGACATC	256	63	(Wuchter et al., 2006)
nifH	AAAGGYGGWATCGGYAARTCCACCAC	TTGTTSGCSGCRTACATSGCCATCAT	459	60	(Rösch et al., 2002)
amoA	GGGGTTTCTACTGGTGGT	CCCCTCKGSAAAGCCTTCTTC	491	56	(Rotthauwe et al., 1997)
nosZ	CGYTGTTCMTCGACAGCCAG	CATGTGCAGNGCRTGGCAGAA	700	64	(Rösch et al., 2002)
narG	TAYGTSGGSCARGARAA	TTYTCRTACCABGTBGC	650	59	(Philippot et al., 2002)

Table S4 The influence of activation of the SA signalling pathway on the diversity of microbial communities in bulk soil and rhizosphere.

	Solonetz				Calcisol			
	rhizosphere		bulk soil		rhizosphere		bulk soil	
Diversity	control	SA	control	SA	control	SA	control	SA
Pridicated Chao 1	2725±135	2785±78	2114±62	2157±59	26492±1987	24986±829	31406±519	30802±485
Observed OUT (richness)	970±46	1025±56	941±18	976±24	2794±124	2681±69	3512±30	3467±35
Simpson diversity index	0.98±0.01	0.98±0.01	0.99±0.00	0.99±0.00	0.98±0.01	0.98±0.01	1	1

Table S5 The abundances of functional genes in bulk soil 72 h after SA treatment. '-' indicates that no gene expression was detected (either Ct values > 40 or no gene amplification was detected). Standard deviations of the mean (n=3) are shown.

	Solonetz		Calcisol	
Genes	Control	SA	Control	SA
arch-amoA	228.9±27.3	274.3±9.9	26.67±7.60	23.79±4.6
ChitinaseA	3267.1±742.2	2160.5±349.8	4488.9±430.9	4384.7±822.0
amoA	30.2±6.7	38.9±5.7	4.5±1.2	6.2±2.3
nifH	19491.3±1192.1	20597.3±1887.9	16311.9±849.2	15468.9±1889.6
narG	2.0±1.2	0.49±0.14	-	-
nosZ	14.9±3.2	8.7±0.60	10.3±0.86	11.6±0.9

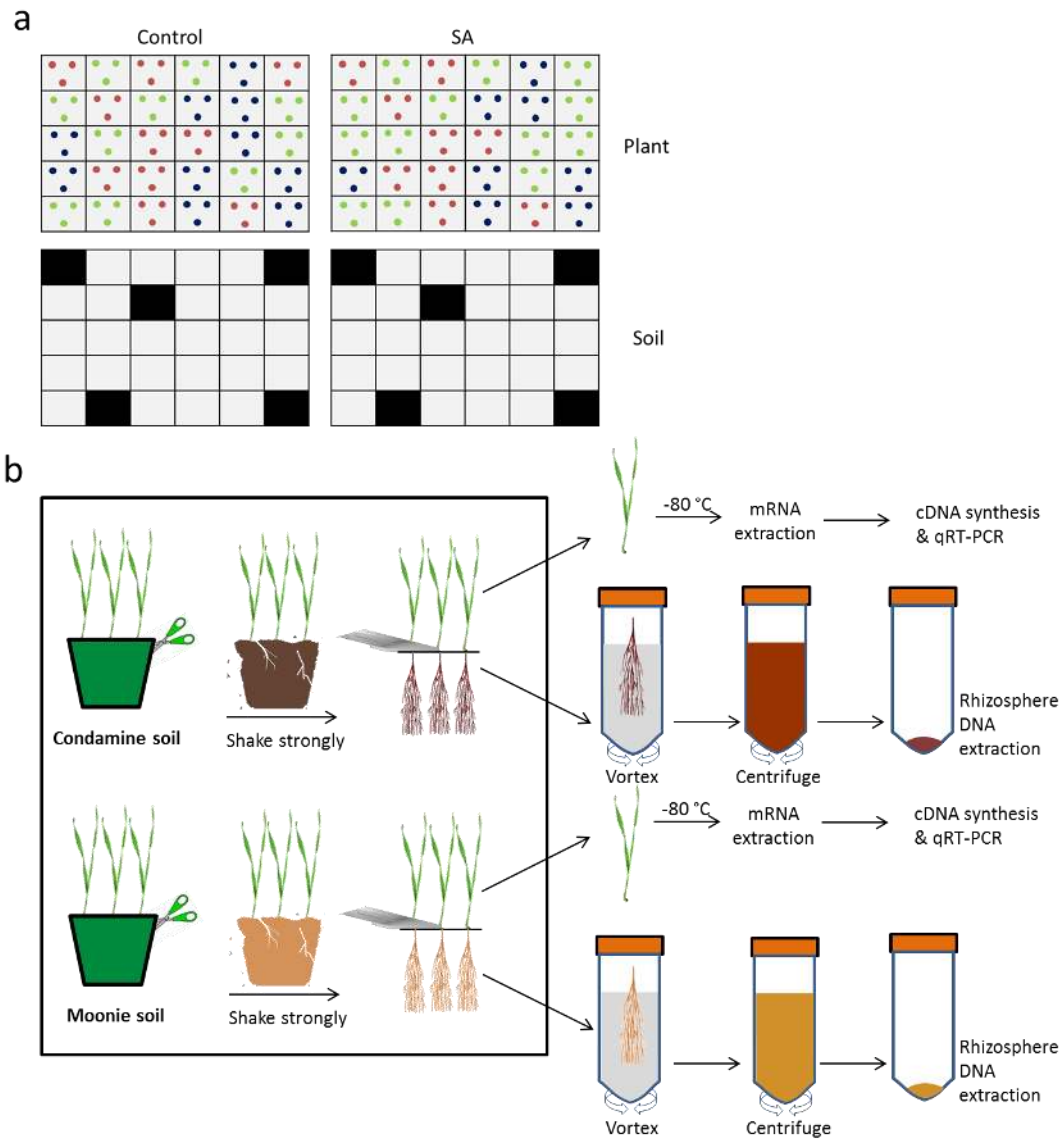
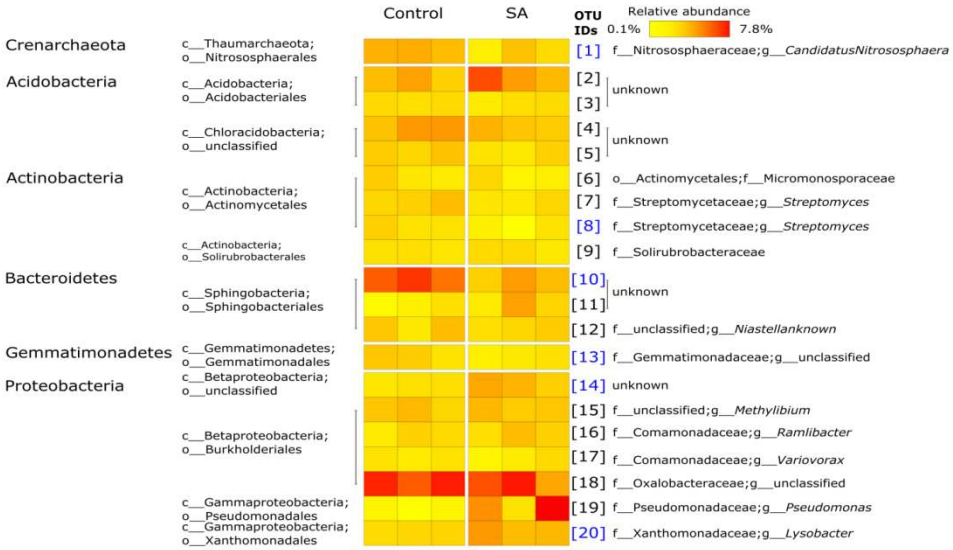
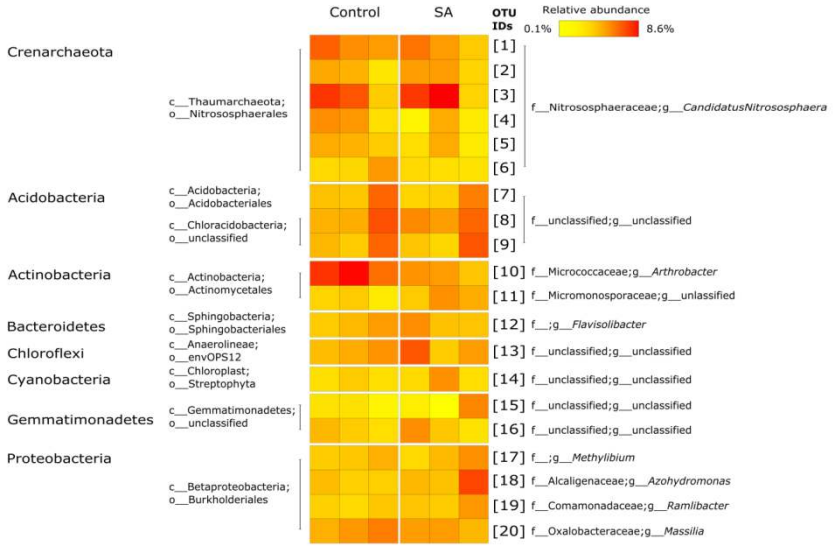


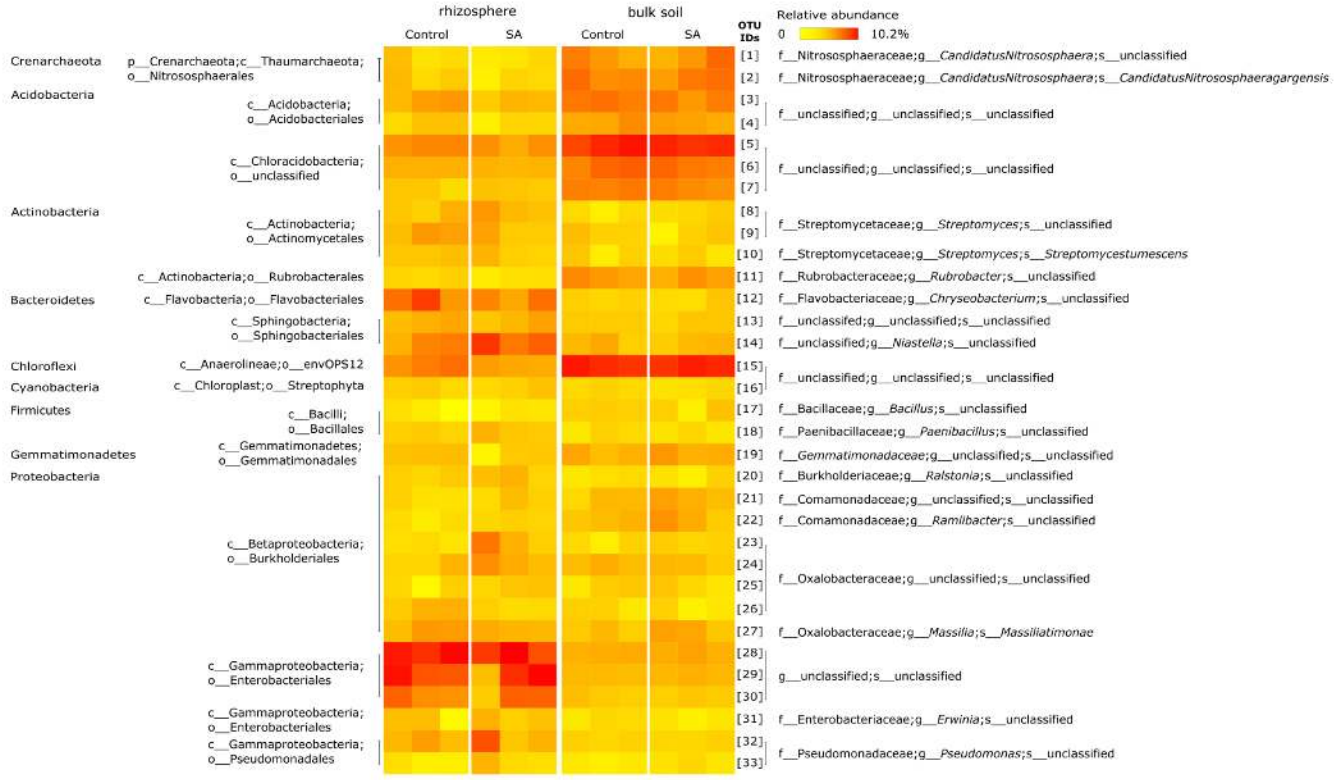
Fig. S1 Schematic figure showing the experiment design (a) and sampling strategy (b). In panel a the four grids represent punnet trays. Half are used for plants which are represented by red, green and blue dots that indicate different bioreplicates. The other trays were used for bulk soil and were only partly filled as indicated by the black squares. Panel b shows the sampling methods, which correspond to the discriptions in the Materials and Methods of this study. Breifly, plants were carefully uprooted from each small pot and shaken vigorously to eliminate bulk soil and the closely attached soils on roots are rhizosphere. The sampling procedures for the rhizosphere soil are detailed in the Materials and Methods.


Control
SA
OTU IDs
Relative abundance
0.1%
7.8%
Crenarchaeota
c__Thaumarchaeota; o__Nitrososphaerales
[1] f__Nitrososphaeraceae;g__CandidatusNitrososphaera
Acidobacteria
c__Acidobacteria; o__Acidobacteriales
[2]
[3]
unknown
c__Chloracidobacteria; o__unclassified
[4]
[5]
unknown
Actinobacteria
c__Actinobacteria; o__Actinomycetales
[6] o__Actinomycetales;f__Micromonosporaceae
[7] f__Streptomycetaceae;g__Streptomyces
[8] f__Streptomycetaceae;g__Streptomyces
c__Actinobacteria; o__Solirubrobacterales
[9] f__Solirubrobacteraceae
Bacteroidetes
c__Sphingobacteria; o__Sphingobacteriales
[10]
[11]
unknown
[12] f__unclassified;g__Niastellanknown
Gemmatimonadetes
c__Gemmatimonadetes; o__Gemmatimonadales
[13] f__Gemmatimonadaceae;g__unclassified
Proteobacteria
c__Betaproteobacteria; o__unclassified
[14] unknown
[15] f__unclassified;g__Methylibium
c__Betaproteobacteria; o__Burkholderiales
[16] f__Comamonadaceae;g__Ramlibacter
[17] f__Comamonadaceae;g__Variovorax
[18] f__Oxalobacteraceae;g__unclassified
c__Gammaproteobacteria; o__Pseudomonadales
[19] f__Pseudomonadaceae;g__Pseudomonas
c__Gammaproteobacteria; o__Xanthomonadales
[20] f__Xanthomonadaceae;g__Lysobacter

a


Control
SA
OTU IDs
Relative abundance
0.1%
8.6%
Crenarchaeota
c__Thaumarchaeota; o__Nitrososphaerales
[1]
[2]
[3]
[4]
[5]
[6]
f__Nitrososphaeraceae;g__CandidatusNitrososphaera
Acidobacteria
c__Acidobacteria; o__Acidobacteriales
[7]
c__Chloracidobacteria; o__unclassified
[8]
[9]
f__unclassified;g__unclassified
Actinobacteria
c__Actinobacteria; o__Actinomycetales
[10] f__Micrococcaceae;g__Arthrobacter
[11] f__Micromonosporaceae;g__unlassified
Bacteroidetes
c__Sphingobacteria; o__Sphingobacteriales
[12] f__;g__Flavisolibacter
Chloroflexi
c__Anaerolineae; o__envOPS12
[13] f__unclassified;g__unclassified
Cyanobacteria
c__Chloroplast; o__Streptophyta
[14] f__unclassified;g__unclassified
Gemmatimonadetes
c__Gemmatimonadetes; o__unclassified
[15] f__unclassified;g__unclassified
[16] f__unclassified;g__unclassified
Proteobacteria
[17] f__;g__Methylibium
c__Betaproteobacteria; o__Burkholderiales
[18] f__Alcaligenaceae;g__Azohydromonas
[19] f__Comamonadaceae;g__Ramlibacter
[20] f__Oxalobacteraceae;g__Massilia

b



c

Fig. S2 Heatmap summarises the relative abundances of operational taxonomic units (OTU) that were present at more than 1% in microbial communities in mock-treated (control) and SA-treated samples. **(a)** Rhizosphere soil of wheat grown in a Solonetz, **(b)** Solonetz nonrhizosphere soil, **(c)** Calcisol rhizosphere and non-rhizosphere soil. The numbers in square brackets indicate OTU numbers that are consistent with those in figures and text. OTUs with statistically significant differences in abundance between control and SA treated samples were depicted in blue in figure a.

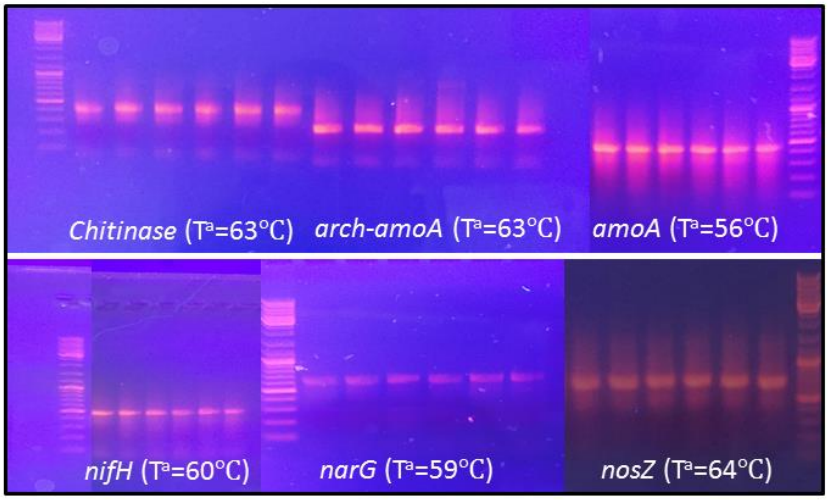
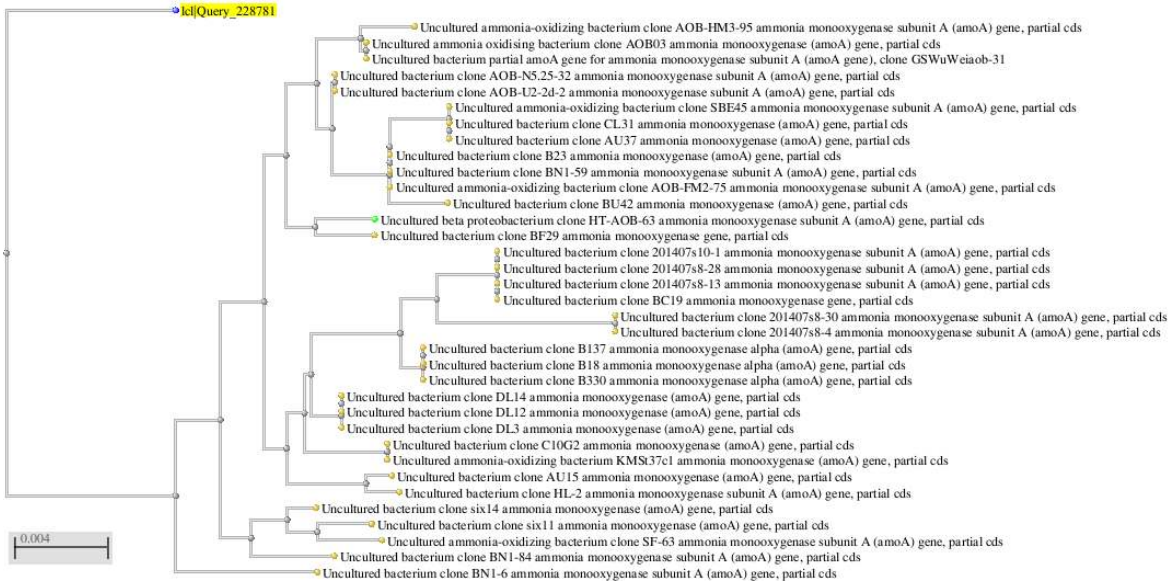
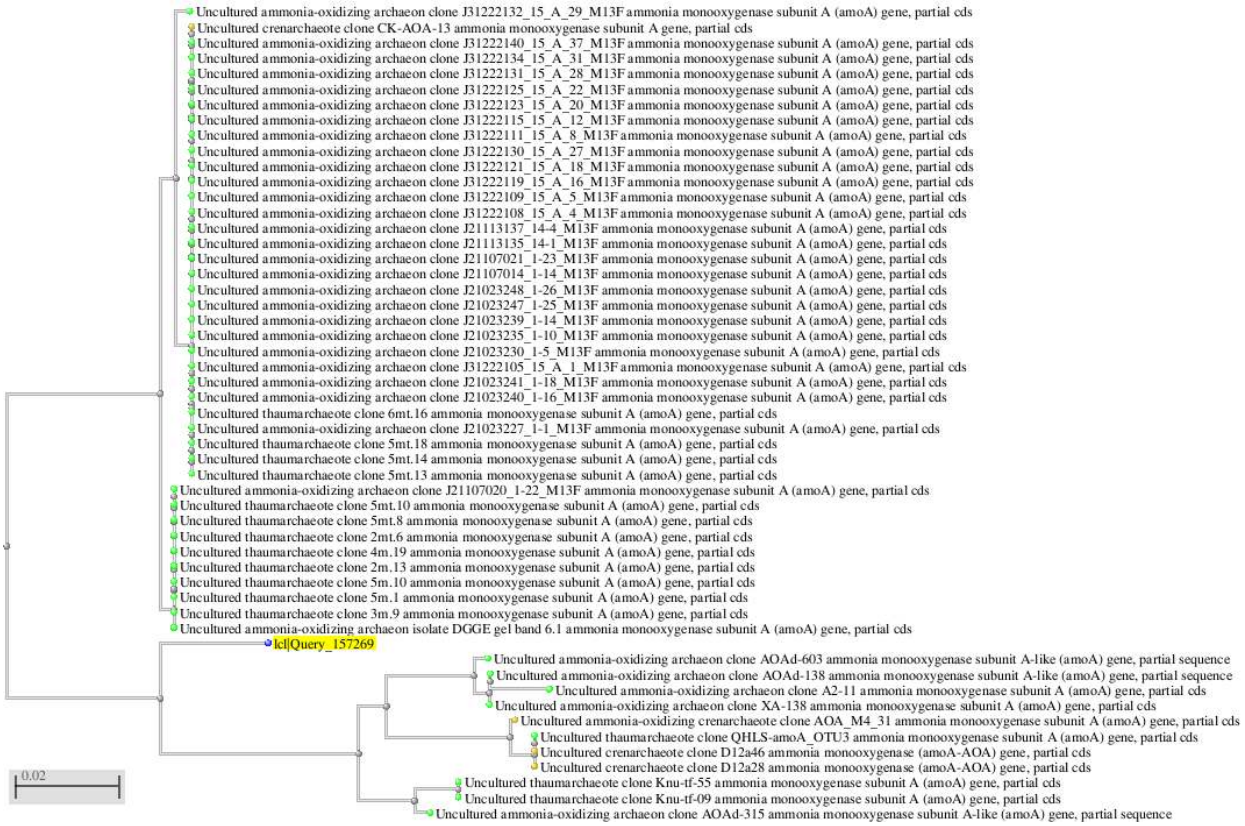


Fig. S3 Verification of primer specificity for the six rhizosphere soil samples using agarose gel (1.5%) electrophoresis and the qPCR products showing single amplicons for *Chitinase group A* (400 bp), *arch-amoA* (256 bp), *amoA* (491 bp), *nifH* (459 bp), *narG* (650 bp) and *nosZ* (700 bp). The 1 Kbp ladder was used as a marker (Fermentas Scientific).



(A)


Uncultured ammonia-oxidizing archaeon clone J31222132_15_A_29_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured crenarchaeote clone CK-AOA-13 ammonia monooxygenase subunit A gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222140_15_A_37_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222134_15_A_31_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222131_15_A_28_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222125_15_A_22_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222123_15_A_20_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222115_15_A_12_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222111_15_A_8_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured ammonia-oxidizing archaeon clone J31222130_15_A_27_M13F ammonia monooxygenase subunit A (amoA) gene, partial cds
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Uncultured thaumarchaeote clone QHLS-amoA_OTU3 ammonia monooxygenase subunit A (amoA) gene, partial cds
Uncultured crenarchaeote clone D12a46 ammonia monooxygenase (amoA-AOA) gene, partial cds
Uncultured crenarchaeote clone D12a28 ammonia monooxygenase (amoA-AOA) gene, partial cds
Uncultured thaumarchaeote clone Knu-tf-55 ammonia monooxygenase subunit A (amoA) gene, partial cds
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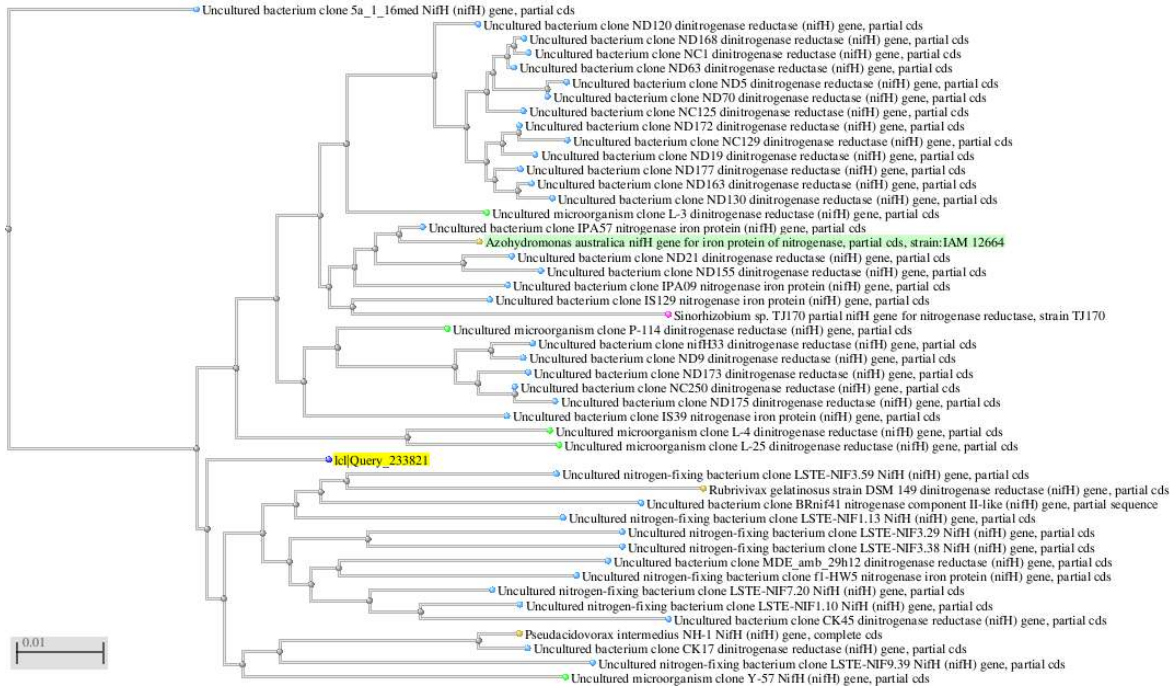
(B)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Uncultured bacterium clone NC129 dinitrogenase reductase (nifH) gene, partial cds
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Uncultured bacterium clone ND163 dinitrogenase reductase (nifH) gene, partial cds
Uncultured bacterium clone ND130 dinitrogenase reductase (nifH) gene, partial cds
Uncultured microorganism clone L-3 dinitrogenase reductase (nifH) gene, partial cds
Uncultured bacterium clone IPA57 nitrogenase iron protein (nifH) gene, partial cds
Azohydromonas australica nifH gene for iron protein of nitrogenase, partial cds, strain:IAM 12664
Uncultured bacterium clone ND21 dinitrogenase reductase (nifH) gene, partial cds
Uncultured bacterium clone ND155 dinitrogenase reductase (nifH) gene, partial cds
Uncultured bacterium clone IPA09 nitrogenase iron protein (nifH) gene, partial cds
Uncultured bacterium clone IS129 nitrogenase iron protein (nifH) gene, partial cds
Sinorhizobium sp. TJ170 partial nifH gene for nitrogenase reductase, strain TJ170
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Uncultured bacterium clone ND173 dinitrogenase reductase (nifH) gene, partial cds
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Uncultured bacterium clone IS39 nitrogenase iron protein (nifH) gene, partial cds
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Uncultured nitrogen-fixing bacterium clone LSTE-NIF1.10 NifH (nifH) gene, partial cds
Uncultured bacterium clone CK45 dinitrogenase reductase (nifH) gene, partial cds
Pseudacidovorax intermedius NH-1 NifH (nifH) gene, complete cds
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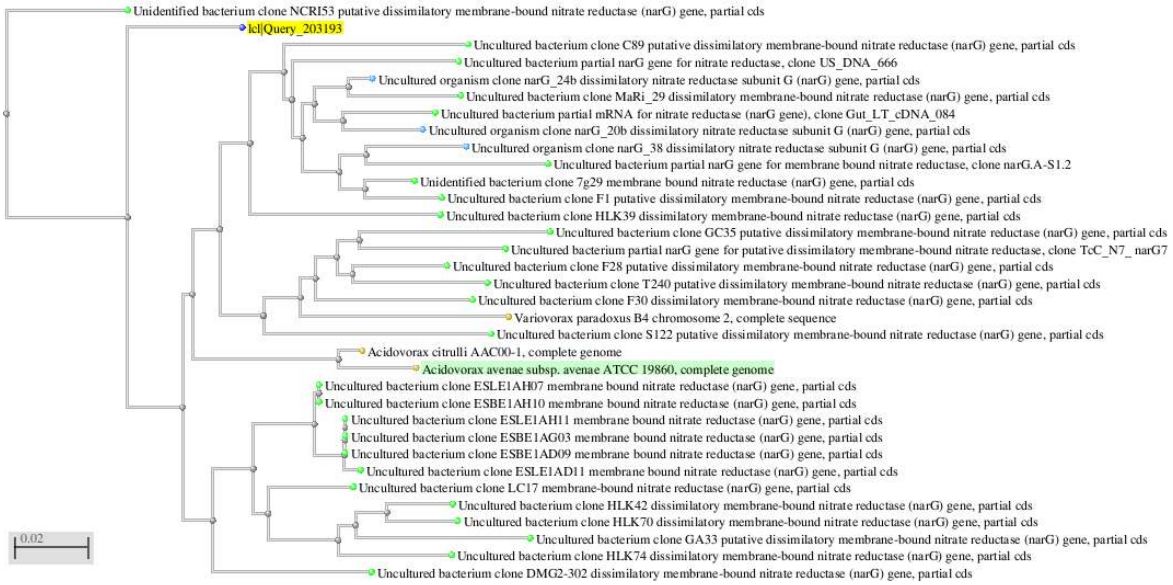
0.01

(C)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Uncultured organism clone narG_24b dissimilatory nitrate reductase subunit G (narG) gene, partial cds
Uncultured bacterium clone MaRi_29 dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
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Uncultured bacterium clone HLK39 dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
Uncultured bacterium clone GC35 putative dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
Uncultured bacterium partial narG gene for putative dissimilatory membrane-bound nitrate reductase, clone TcC_N7_ narG7
Uncultured bacterium clone F28 putative dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
Uncultured bacterium clone T240 putative dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
Uncultured bacterium clone F30 dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
Variovorax paradoxus B4 chromosome 2, complete sequence
Uncultured bacterium clone S122 putative dissimilatory membrane-bound nitrate reductase (narG) gene, partial cds
Acidovorax citrulli AAC00-1, complete genome
Acidovorax avenae subsp. avenae ATCC 19860, complete genome
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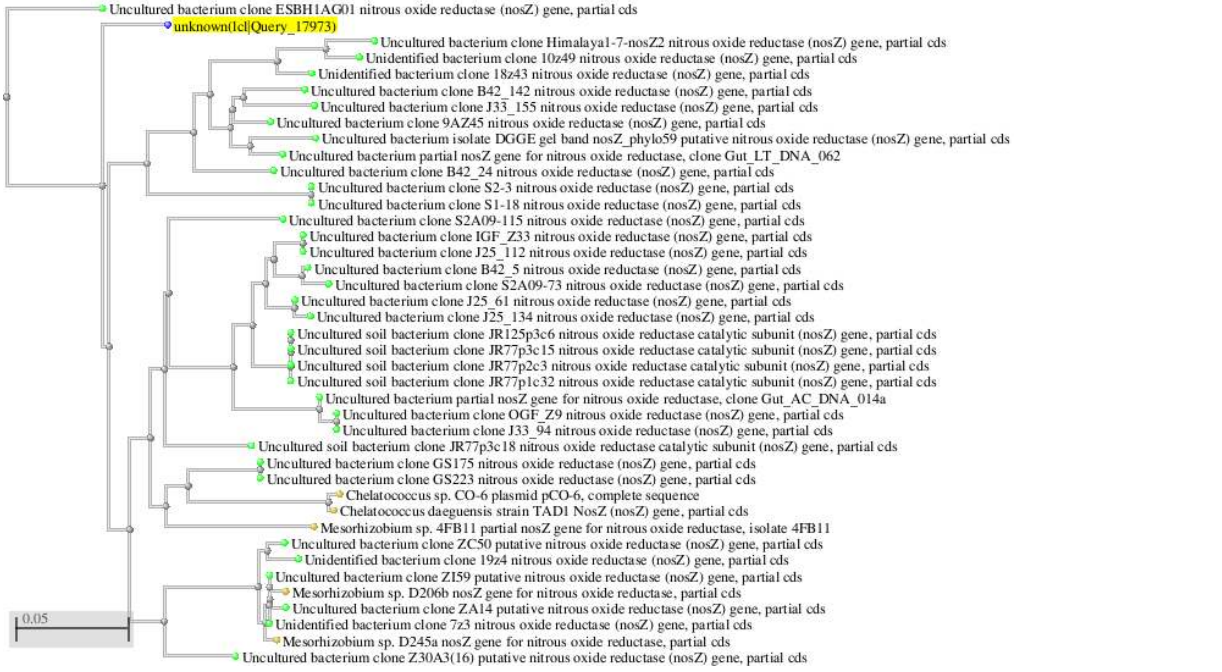
0.02

(D)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Unidentified bacterium clone 18z43 nitrous oxide reductase (nosZ) gene, partial cds
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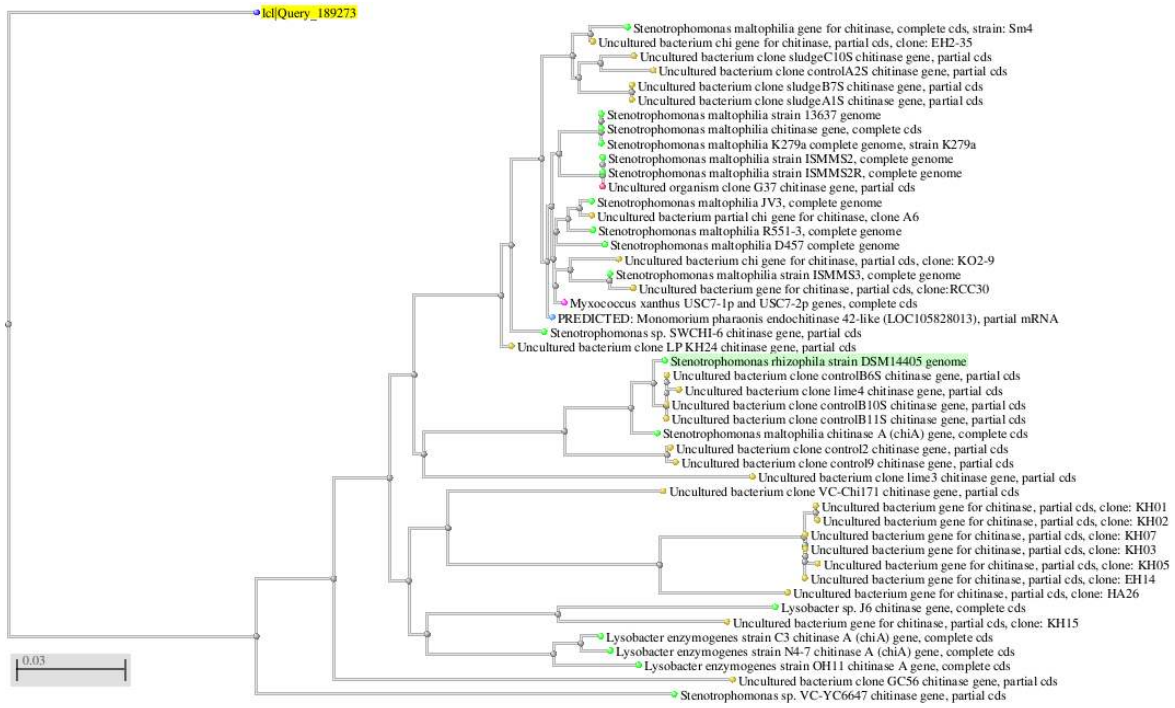
Uncultured bacterium clone J33_155 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone 9AZ45 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium isolate DGGE gel band nosZ_phylo59 putative nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium partial nosZ gene for nitrous oxide reductase, clone Gut_LT_DNA_062
Uncultured bacterium clone B42_24 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone S2-3 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone S1-18 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone S2A09-115 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone IGF_Z33 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone J25_112 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone B42_5 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone S2A09-73 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone J25_61 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone J25_134 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured soil bacterium clone JR125p3c6 nitrous oxide reductase catalytic subunit (nosZ) gene, partial cds
Uncultured soil bacterium clone JR77p3c15 nitrous oxide reductase catalytic subunit (nosZ) gene, partial cds
Uncultured soil bacterium clone JR77p2c3 nitrous oxide reductase catalytic subunit (nosZ) gene, partial cds
Uncultured soil bacterium clone JR77p1c32 nitrous oxide reductase catalytic subunit (nosZ) gene, partial cds
Uncultured bacterium partial nosZ gene for nitrous oxide reductase, clone Gut_AC_DNA_014a
Uncultured bacterium clone OGF_Z9 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone J33_94 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured soil bacterium clone JR77p3c18 nitrous oxide reductase catalytic subunit (nosZ) gene, partial cds
Uncultured bacterium clone GS175 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone GS223 nitrous oxide reductase (nosZ) gene, partial cds
Chelatococcus sp. CO-6 plasmid pCO-6, complete sequence
Chelatococcus daeguensis strain TAD1 NosZ (nosZ) gene, partial cds
Mesorhizobium sp. 4FB11 partial nosZ gene for nitrous oxide reductase, isolate 4FB11
Uncultured bacterium clone ZC50 putative nitrous oxide reductase (nosZ) gene, partial cds
Unidentified bacterium clone 19z4 nitrous oxide reductase (nosZ) gene, partial cds
Uncultured bacterium clone ZI59 putative nitrous oxide reductase (nosZ) gene, partial cds
Mesorhizobium sp. D206b nosZ gene for nitrous oxide reductase, partial cds
Uncultured bacterium clone ZA14 putative nitrous oxide reductase (nosZ) gene, partial cds
Unidentified bacterium clone 7z3 nitrous oxide reductase (nosZ) gene, partial cds
Mesorhizobium sp. D245a nosZ gene for nitrous oxide reductase, partial cds
Uncultured bacterium clone Z30A3(16) putative nitrous oxide reductase (nosZ) gene, partial cds
0.05

(E)



(F)

Fig. S4 Distance tree produced by NCBI BLAST using pairwise alignments for *amoA* (A), *arch-amoA* (B), *nifH* (C), *narG* (D), *nosZ* (E) and *ChitinaseA* (F).

Chapter 7 Conclusion

In Chapter 2, 3 and 4 of this thesis, I tested the effects of ST on the soil physicochemical and biological properties in long-term NT soils in eastern Australia. The implements of chisel, offset disc and Kelly chain provide less soil inversion compared with aggressive tillage implements, such as the mouldboard plough. In general, the tillage effects on soil physicochemical and biological properties as well as agronomic productivity (only tested in the Moree trial) were minor. This is particularly true for the tested grey Vertosol at the Moree site as all soil parameters tested were not influenced by tillage treatments within a short time-frame. This may have been due to the high resistance and resilience of the clay-rich soil type of Vertosol. However, it must be noted that while there were minimal impacts on soil health and agronomic productivity, the weather in the Moree site was dry during the testing period, which may not represent the typical climate in this area. Therefore, further research may be required to assess the potential impacts of the wetting up process on soil properties and productivity.

In the Moonie field trial, one-time ST using two minimal soil inversion implements (chisel or offset disc) did not affect overall soil microbial communities. However, relative to the NT, chisel tillage led to slight increases in microbial biomass carbon, abundances of Alphaproteobacteria, Bacteroidetes and Firmicutes, and the utilisation of D+ cellubiose as well as mannitol at 0-10 cm depth. Therefore, one-time ST in the Moonie site using either chisel or offset disc had a minor positive influence on soil biological attributes of the NT Calcisol. In conjunction with our previous findings that the soil physicochemical properties were not influenced by the one-time ST, Chapter 2 results may suggest that one-time tillage using low soil inversion implements may be suitable to tackle the weeds issues in the NT Calcisol. In the Condamine field trial, the Solonetz tested is a texture contrast soil between layers and the higher number of Acidobacteria in the surface soil (0-10 cm) than the deeper soil (10-20 cm) defined the characteristics of this slightly acidic Solonetz. Interestingly, the Solonetz showed minor increases in Acidobacteria o_iii 1-15 and Acidobacteria o_RB41, and an increase trend in soil microbial activity one year after tillage. However, all the other tested microbial parameters were generally not influenced by ST. These results indicate that ST only caused slight changes in soil microbial community structure but not major changes in microbial properties. Additionally, the results from the Condamine trial also provide comprehensive data on DNA-based abundance of genes related to the nitrogen and carbon cycles along with the microbial communities of the Solonetz. No significant changes in soil functional potentials of nitrogen and carbon cycles were observed. Further long-term studies are needed in this texture contrast soils to

identify the effects of ST on these soil parameters and the possible higher soil total enzymatic activity and microbial biomass.

In conclusion, Chapters 2, 3 and 4 of this thesis demonstrate that ST had no detrimental short-term impacts on soil health. These results indicate that ST can be used as a suitable strategy to address issues faced by growers in NT systems in eastern Australia due to its minimal impacts on soil health and productivity. This thesis research may suggest a place for ST in agricultural systems on the proper usage of tillage implements. Table 1 summarises recommendations made for farmers suggesting which conditions to use ST.

Table 1 Safe implementation of ST in otherwise NT farming systems (adapted from Dang et al. 2016).

Purpose of tillage	Optimum tillage time	Tillage implement	References
Weed management			
In-crop	Prior to weed flowering	Shallow tine	Pratley (2000)
Fallow	Post seed fall, before germinating rains	Disc	McGillion and Storrie (2006)
Nutrient stratification			
Sodic soil	Post-harvest, early in fallow	Para plough	Dang et al. (2010)
non-sodic soil	Post-harvest, early in fallow	Deep ripper tine	Bell et al. (2012)
Soil physical constrains			
Surface soil	Early in fallow	Cross tine	Spoor (2006)
Sub soil	Early in fallow	Deep ripping tine	Hamza and Anderson (2005)

The traditional (e.g., qPCR and T-RFLP) and newly emerging molecular methods of next generation sequencing used in the present tillage research should have been powerful enough to detect soil differences, as soil depth effects have been often detected. The combined use of classical and molecular techniques in this thesis may provide a useful toolbox to measure the impact of disturbances on soil microbial communities. The parameters of microbial activity and community structure should be used conjointly for determining microbial properties of soil samples in the future. It should be also noted that in the present study, only short-term impacts of ST were examined, while studies of longer timeframes should be considered to monitor the long-term ST effects on soil microbial properties.

Hormone homeostasis effects on *Arabidopsis*-associated microbial communities have been examined previously but with inconsistent results being reported (Carvalhais et al. 2013; Doornbos et al. 2011). In Chapter 5 and 6 of this thesis, the effects of the activation of JA and SA signalling pathways on wheat-associated bacterial communities were examined. Overall, my results support the hypothesis that the activation of plant signalling pathways influences the composition and diversity of the wheat microbiome. It was observed that the activation of the JA signalling pathway reduced the diversity and changed the composition of root endophytic bacterial communities. However, the microbial communities in the wheat shoot endosphere or rhizosphere were not influenced. These findings suggest that the effects of JA signalling on the wheat microbiome are specific to individual plant compartments. Further, most of the root endophytic populations that became more abundant in response to JA signalling, were closely related to taxa previously reported to promote plant growth, mobilise nutrients or suppress bacterial, fungal or viral phytopathogens. These results support the notion that an elevated plant defence signalling may not only control phytopathogens but also restrict the colonisation of root endophytic microbial communities. As the Actinomycetes order was greatly influenced by the activation of JA signalling, Actinomycetes could be important in plant response to environmental stresses, e.g., invasion of necrotrophic pathogens that are controlled by the JA pathway.

The activation of the SA signalling pathway also led to changes in wheat-associated microbial communities. Those archaeal/bacterial components involved in N cycling were decreased in the rhizosphere of wheat that was cultivated in Solonetz but not in Calcisol. These results provide evidence that soil type and nutrient conditions may influence microbial community response to the activation of the SA signalling pathway in wheat. However, further studies are warranted to reveal the mechanisms behind this phenomenon. Plants are sessile and have developed cost-efficient response mechanisms under undesirable environmental conditions. Accordingly, the increase in *Lysobacter* spp. and the decreased abundance in the N cycling components in the rhizosphere by the SA treatment in the present study may support this conception. Sphingobacteria that produce sphingolipids reduced in abundance upon the enhanced SA signalling, which may call for future studies to reveal the possible functional consequences on wheat. Future experiments can investigate the effects of JA and SA signalling on the functions of wheat-associated microbiomes by using the function-based metagenomics analysis.

References

Bell M, Lester D, Smith L, Want, P. (2012) Increasing complexity in nutrient management on clay soils in the northern grain belt-nutrient stratification and multiple nutrient limitations. In: Yunusa I (Hrsg.), Capturing Opportunities and Overcoming Obstacles in Australian Agronomy. Proceedings of 16th Australian Agronomy Conference 2012, 14-18 October 2012, Armidale, NSW. http://www.regional.org.au/au/asa/2012/nutrition/8045_bellm.htm#TopOfPage.

Carvalhais, LC, Dennis PG, Badri DV, Tyson GW, Vivanco, JM, Schenk, PM. (2013) Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. PLoS One 8, e56457 (2013).

Dang YP et al. (2010) Diagnosis, extent, impacts, and management of subsoil constraints in the northern grains cropping region of Australia. Australian Journal of Soil Research 48: 105-119.

Doornbos, RF, Geraats, BP, Kuramae, EE, Van Loon, L. & Bakker, PA. (2011) Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. Molecular Plant-Microbe Interactions 24, 395-407.

Hamza, MA, Anderson, WK. (2005) Soil compaction in cropping systems: A review of the nature, causes and possible solutions. Soil and Tillage Research 82: 121-145.

McGillion, T, Storrie, A. (2006) Integrated weed management in Australian cropping systems: a training resource for farm advisors. Cooperative Research Centre for Australian Weed Management, Adelaide, Australia.

Pratly JE. (2000) Tillage and other physical management methods. In: Sindel BM (Editor), Australian Weed Management Systems, Melbourne.

Spoor, G. (2006) Alleviation of soil compaction: Requirements, equipment and techniques. Soil Use and Management 22: 113-122.

Future aspects

- It would still be required to determine if the application of ST influences fungal communities of the long-term NT soils;
- In the present study, only short-term impacts of ST were examined while longer timeframes, for instance a 5 years' study should be considered to monitor the long-term ST effects on soil microbial properties;
- To apply the conclusions more broadly, it would be necessary to examine ST effects on the soil microbial properties in other grain growing regions of different soil types under different climatic conditions;
- It would be worth testing to use a specific plant disease to examine how wheat associated microbial communities respond to the alterations in plant defence signalling modes;
- Hormone signalling effects on the plant associated microbial community can be examined over longer time frames after SA/JA treatment (e.g., 6 days or a time course) as it may take longer time for microbial communities to respond to hormone treatments;
- The influence of hormone signalling effects on the fungal communities in wheat rhizosphere and endosphere should be tested;
- Different soil types should also be examined to test hormone signalling effects on wheat-associated microbial communities;
- Changes in protein profiles in rhizosphere after the SA and JA treatments can be investigated using proteomic methods (meta-proteomics approach);
- Further work should also consider integrative approaches using plant mutants and functional metatranscriptomic/metagenomic analyses to reveal further roles of plant interactions with associated bacterial communities;
- In the long term, wheat crop yields may be improved and protected against biotic and abiotic stresses by engineering wheat-optimised microbiomes or by breeding wheat cultivars with improved microbiome interactions.

Appendix 1 "Inner Plant Values: Colonization, Diversity and Benefits from Endophytic Bacteria" (Submitted to Critical Reviews in Microbiology, BMCB-2016-0137).

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Abstract

Plants host a considerable number of endophytic bacteria inside their tissues whose role in plant growth, development and resistance to biotic and abiotic stresses is being increasingly recognized. A picture is emerging where plant roots act as gatekeepers to screen soil bacteria from the rhizosphere and rhizoplane. This typically results in endophytic microbiomes dominated by Proteobacteria, Actinobacteria and to a lesser extent Bacteroidetes and Firmicutes, but that are depleted of Acidobacteria. Motility, plant cell-wall degradation ability, and reactive oxygen species scavenging seem to be crucial factors for successful endophytic colonization and establishment. Due to their plant growth-promoting traits, endophytic bacteria are being widely explored for their use in the improvement of crop performance. Some endophytes elegantly endow plant priming conditions which elicit a faster and stronger defense once pathogens attack. Overall, insights into the mechanism of endophytic bacterial colonization and interactions with plants may help us manipulate endophytic microbiomes for improving agricultural production. In this review, based on the most recent studies, we aim to discuss (1) where and how bacterial endophytes colonize plants; (2) how endophytic bacteria respond to plant defense signaling; and (3) how endophytic traits influence plant growth and resistance to biotic and abiotic stresses.

Keywords

Biocontrol bacteria; endophytic bacteria; plant defense signaling; plant growth promotion; plant microbiome.

1 Introduction

Endophytes are non-pathogenic organisms that live inside plant tissues for at least part of their life cycles (Rosenblueth & Martínez-Romero, 2006). Endophytes are found in all plants and are dominated by bacteria and fungi. The number of bacterial cells within endophytic environments reaches c. 10^4-10^8 per gram of plant tissue, which is considerable even when compared with the rhizosphere (c. 10^6-10^9 bacterial cells g^{-1} plant tissue) and bulk soil (c. 10^6-10^9 bacterial cells g^{-1} soil) (Bulgarelli et al., 2013). Root endophytes are generally recruited from soil and can spread systemically to stems and leaves via the apoplast in xylem vessels (Chi et al., 2005). Other routes of colonization include access through natural openings in the phyllosphere, e.g., stomata on leaves and lenticels of a woody plant (Vorholt 2012), or vertical transmission from seeds (Truyens et al., 2015). Collectively, endophytes influence plant health and nutrition (Compant et al., 2005a). A better understanding of their ecology may facilitate optimization of these communities for improved crop production.

A common technical challenge is to effectively separate endophytes from epiphytes (e.g. those bacteria on rhizoplane and leaf surface). Surface sterilization by disinfecting with sodium hypochlorite and ethanol as well as mechanical removal of microbes closely attached to the root surface by vigorous shaking with glass beads or ultrasonication have been used for this purpose (Bulgarelli et al., 2012; Lundberg et al., 2012; Reinhold & Hurek, 1989). The chemical disinfection seems to be more effective but may underestimate the presence of bacteria as it damages DNA (Reinhold-Hurek et al., 2015). A diverse range of bacteria have been isolated from surface-sterilized plant tissues, such as roots, tubers, stems, leaves, seeds, flowers, fruits and legume nodules (Compant et al., 2011; Deng et al., 2011; Marques et al., 2015; Rosenblueth & Martínez-Romero, 2006; Truyens et al., 2015). These isolates are considered to be endophytes and include representatives of the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Rosenblueth & Martínez-Romero, 2006). Occasionally, such isolates also include bacterial food pathogens, such as *Escherichia coli* (Wright et al., 2013) and *Clostridium botulinum* (Zeiller et al., 2015), and even *Burkholderia pseudomallei*, which is a known human pathogen (Prasertsincharoen et al., 2015). Many of these endophytic isolates can promote plant growth, such as *Burkholderia* sp. strain PsJN and *Azoarcus* sp. strain BH72, which have been isolated from onion (*Allium cepa* L.) and kallar grass (*Leptochloa fusca* L. Kunth) roots, respectively (Frommel et al., 1991; Hurek & Reinhold-Hurek, 2003). Advances in culture-independent molecular methods, such as next generation sequencing (NGS) and PhyloChip technology, have greatly increased our understanding of the structure and function of plant microbiomes (Bulgarelli et al., 2012; Mendes et al., 2011).

Consistent with culture-based methods, Proteobacteria and Actinobacteria were observed to be the dominant taxa in the endophytic bacterial communities using NGS (Bulgarelli et al., 2012).

During millions of years of coevolution with soil microbes, plants have developed a diverse range of mechanisms to cope with abiotic and biotic stresses. Establishing continuing relationships with endophytic bacteria may enhance the plant's capability to defend themselves against stresses and potentially get benefits for growth and development. For instance, endophytic bacteria are able to produce phytohormones such as gibberellins (GAs) and indole acetic acid (IAA) which promote plant growth (Khan et al., 2014). In addition, some endophytic bacteria can systemically prime the plant's immune system. Primed plants do not display major changes in defense-related gene expression in the absence of a pathogen, but upon pathogen or insect attack display an accelerated defense response, providing broad-spectrum resistance (Conrath et al., 2015; Pieterse et al., 2014). It was recently found that endophytic bacteria can be directly digested by plant cells to be used as a nitrogen (N) source (Beltran-Garcia et al., 2014). Endophytic bacteria can also protect plants from a series of abiotic stresses, such as drought (Rolli et al., 2015; Sheibani-Tezerji et al., 2015), low temperature (Su et al., 2015; Subramanian et al., 2015), and salinity (Ali et al., 2014).

In this review, we aim to discuss important issues regarding the interactions between plants and endophytic bacteria. We ask (1) which bacteria live in plant endophytic habitats, (2) how do endophytic bacteria respond to plant stresses and environmental stimuli, (3) where do endophytic bacteria colonize plants, (4) what are the traits that endow endophytic bacteria for the invasion and infection inside plants, (5) how do endophytes deal with plant immunity, (6) how does the plant host influence endophytic colonization via hormone signaling pathways, and (7) what are the promising traits of endophytic bacteria that make them interesting for applications in sustainable agriculture. We believe that understanding the interactions between endophytic bacteria and their hosts will assist in the design of new strategies for productive and sustainable practices in agriculture.

2 Biodiversity of Endophytic Bacteria

The plant interior harbors bacterial microbiomes with lower abundance and diversity than the rhizosphere (Bulgarelli et al., 2012). The taxonomic structure of bacterial communities in plant endophytic compartments is dominated by the phyla Proteobacteria and Actinobacteria (Bulgarelli et al., 2012; Carrell & Frank, 2015; Lebeis et al., 2015; Lundberg et al., 2012; Mora-Ruiz et al.,

2015; Yu et al., 2015). Firmicutes, Bacteroidetes, Gemmatimonadetes, Verrucomicrobia, Planctomycetes, Fusobacteria and some the other bacterial phyla may also be present but in lower abundances (Edwards et al., 2015; Sessitsch et al., 2012). In contrast, Archaea and Acidobacteria are totally depleted from these habitats (Sessitsch et al., 2012).

Gammaproteobacteria of the genera *Enterobacter* and *Pseudomonas* are the dominant endophytic bacteria on different plants, including tuberous roots of sweet potato (Marques et al., 2015), rice roots (Ferrando & Scavino, 2015; Ren et al., 2015a; Sessitsch et al., 2012), and the roots of mature trees of *Populus deltoids* (Gottel et al., 2011). The predominance of one or two operational taxonomic units (OTUs) in specific plant tissues has been reported after endophytic bacterial sequencing, such as a pseudomonas-like OTU in the roots of *P. deltoids* (Gottel et al., 2011) and two OTUs affiliated to *Pseudomonas* and *Enterobacter* in sugarcane stems (Magnani et al. 2013). Using cultivation-based methods, it was observed that the *Enterobacter oryziphilus* sp. nov. and *Enterobacter oryzendophyticus* sp. nov. were the main bacterial inhabitants in the rice root endosphere. Inoculation of rice with these two bacteria demonstrated plant growth promoting effects on rice growth via improving N and P nutrition (Hardoim et al., 2013). These studies support the hypothesis that plants co-evolve with endophytes and actively select beneficial bacteria, which results in the overwhelming dominance of some taxa inside plants. The *Streptomycetaceae* family dominated the Actinobacteria phylum members in the endophytic compartment of *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012).

The leaf and root endophytic bacterial microbiomes have a significant overlap in both taxonomy and function, with the major groups being Proteobacteria, Actinobacteria, and Firmicutes (Bai et al., 2015; Bodenhausen et al., 2013; Bulgari et al., 2014). For instance, these taxa were found to be the dominant phyla in the leaves of common bean (*Phaseolus vulgaris*) using a culture-based approach (de Oliveira Costa et al., 2012). Given that many plant growth promoting bacteria belong to these taxa and have been reported to be competent colonizers of plant tissues, endophytic bacteria hold great potential as targets in screening studies aiming at beneficial bacteria for crop growth or disease control.

The abovementioned studies collectively demonstrate that root endophytic bacteria are distinct assemblages rather than random subsets of the rhizosphere. Two- and three-step models for the plant's recruitment of bacteria have been proposed (Bulgarelli et al., 2013; Reinhold-Hurek et al., 2015). It seems that the ectorhizosphere, rhizoplane and plant immune system may serve as the first, second and the third screening points, respectively. Bacteria lacking motility, chemotaxis, pili

structures or adhesion ability may not be able to colonize on the rhizoplane properly (Reinhold-Hurek et al., 2015). In addition, the plant immune system may actively exclude specific bacteria. The special conditions in the ectorhizosphere and roots may greatly hinder the colonization by Acidobacteria and Archaea. However, the mechanisms underlying the depletion of Acidobacteria and Archaea from inside the plants and the ecological rationale behind this phenomenon are still unknown. It is clear that the bacterial diversity in the plant endosphere is dynamic and is affected by many different plant factors, which are discussed in the following sections.

3 Factors Driving Endophytic Bacterial Communities

Plant microbiomes may contribute to plant growth and resistance to phytopathogens and herbivores in direct and/or indirect ways (Upreti & Thomas, 2015). Endophytic bacterial microbiomes may vary according to host plant species (Ding & Melcher, 2016; Shen & Fulthorpe, 2015), plant genotype (Marques et al., 2015; Rodriguez-Blanco et al., 2015), plant organs (Hameed et al., 2015), plant developmental stage (e.g. seedling or mature plant) (de Almeida Lopes et al., 2016; Ren et al., 2015a; Yu et al., 2015), growing season (e.g. trees) (Ding & Melcher, 2016; Shen & Fulthorpe, 2015), geographical location (field conditions) (Edwards et al., 2015), soil type (Edwards et al., 2015), host plant nutrient status (Hameed et al., 2015), cultivation practice (Edwards et al., 2015) and fertilization (Rodriguez-Blanco et al., 2015). Nevertheless, characteristics of the plant host seem to be the main drivers in shaping the endophytic microbiome. In *Arabidopsis* and rice a variety of endophytic bacterial groups were shared amongst different soils (Edwards et al., 2015; Lundberg et al., 2012). Furthermore, alpha and beta diversity of endophytic bacteria in rice were only genotype- but not soil type-dependent at the phylum scale (Edwards et al., 2015). Genetically modified plants also differ in colonization by endophytic bacterial communities. This was observed with transgenic glyphosate-resistant cultivars of soybean that had a higher abundance and diversity of culturable endophytic bacteria than wild type plants (de Almeida Lopes et al., 2016). Interestingly, the plant genotype may also affect the functional diversity of endophytic bacteria as IAA-producing strains were predominantly isolated from one of the three genotypes of sweet potato studied (Marques et al., 2015).

Biotic stresses such as plant diseases can also influence the composition of endophytic bacterial communities. An anaerobic pectolytic *Clostridia* population was particularly enriched in potato (*Solanum tuberosum*) tubers upon infection by *Pectobacterium atrosepticum*, the soft rot disease causing agent (Koiv et al., 2015). This change in infected plants possibly occurred due to oxygen depletion inside the tubers (Koiv et al., 2015).

In addition to the host properties, abiotic factors, including fluctuations of CO_2 and temperature also influence endophytic bacterial communities. In the context of climate change and given the importance of endophytic bacteria for plant growth and health, understanding how endophytic bacteria respond to elevated CO_2 and temperature helps decision-making policies around environmental issues. Compared with soil bacterial communities, leaf endophytic bacteria seem to be more vulnerable to climate change (Ren et al., 2015b). For example, the community structure of endophytic bacteria in rice leaves was influenced by elevated CO_2 levels at the tillering and filling stages, but not during maturity, and this influence also correlated with N fertilization levels (Ren et al., 2015a). Moreover, endophytic communities inhabiting leaves at different locations in the plant (upper or lower leaf) respond differentially to elevated CO_2. Oxygen availability also exerts effects on endophytic bacterial communities in rice, especially on diazotrophs (Ferrando & Scavino, 2015). For instance, diazotrophic community composition was shifted remarkably by flooding, with Gammaproteobacteria and Betaproteobacteria being predominant in rice roots before and after flooding, respectively. The gene involved in N fixation (*nifH*) was more abundant after flooding (Ferrando & Scavino, 2015).

Compared with taxonomy-based approaches used in the abovementioned studies, function-based metagenomic analysis has more potential to represent the functional variations of endophytic communities. Approaches to harness functional changes in endophytic communities for plant stress alleviation can only be developed if mechanisms that trigger such changes are better understood. However, investigations on the functional changes of endophytic communities have been performed to a much lesser extent than phylogeny-based analyses. Recently, a functional study conducted on tomato plants revealed that bacterial endophytes colonizing roots were significantly affected by the root-knot nematode and genes involved in plant polysaccharide degradation, carbohydrate/protein metabolism, and N_2 fixation were increased in abundance (Tian et al., 2015). This observation provides evidence to suggest that particular functional attributes of endophytic bacteria are induced upon stress suffered by plants.

4 Distribution of Endophytic Bacteria and Colonization Patterns

Bacterial colonization patterns in plant endophytic compartments have thus far been mainly studied in grasses (e.g. rice and kallar grass) using cultivated model strains. Some of the most popular approaches for such evaluation include fluorescence *in-situ* hybridization (FISH) and

genetically engineered bacterial strains tagged with reporter genes (e.g. *gfp* or *gus*) combined with microscopy to enumerate and visualize colonization of endophytic bacteria in plant tissues.

In most plants, the plant parts close to soil harbor more bacteria than the uppermost plant organs (Fisher et al., 1992). Lateral root emergence sites are usually hot spots for bacterial colonization (Reinhold-Hurek et al., 2006). Emerging lateral roots break through the epidermis, cortex, endodermis, casparian strip (band around endodermis) and pericycle, thereby naturally forming a 'highway' for bacteria to enter at these sites. From there, bacteria can further enter the phloem and xylem vessels that transport photosynthates (phloem), nutrients and water (xylem). Endophytic bacteria are typically detected in outer cell layers, root cortex, phloem and xylem, in the apoplast as well as intracellularly. Bacteria colonizing inside the root conductive tissues can be further transported to shoots and leaves driven by plant transpiration (Compant et al., 2010). Endophytic infection can also occur at wounds (e.g., leaf scars, root ruptures) as a result of herbivore damage (Compant et al., 2010). Typical colonization sites of endophytic bacteria are schematically represented in **Fig. 1**. For instance, the diazotrophic bacterial strain *Paenibacillus polymyxa* P2b-2R extensively colonizes the surface and inside of roots, stems and needles of lodgepole pine (*Pinus contorta* Dougl. var. latifolia Engelm.), primarily intracellularly but also in the apoplast (Anand & Chanway, 2013; Anand et al., 2013). The plant growth promoting bacteria *Burkholderia* sp. strain PsJN colonizes root rhizodermis cells, internal tissues, particular internodes and leaves of grapevine (Compant et al., 2005b; 2008). An unusual colonization strategy has been recently discovered for the facultative intracellular symbiont *Methylobacterium extorquens* strain DSM13060, which aggregated around the nucleus of the living cells of Scots pine (*Pinus sylvestris* L.) shoot tips (Koskimäki et al., 2015).

Endophytic bacterial colonization can be categorized into 'obligate', 'facultative' and 'passive' depending on if it requires plant tissue to live and reproduce (for a review on this topic see Hardoim et al., 2008). Obligate endophytic bacteria are derived from seeds and cannot survive in soils. Facultative endophytic bacteria widely exist in soil, and they carry out colonization and infection when conditions are suitable. Most facultative endophytic bacteria remain within the cortex but some also enter central phloem and xylem (Compant et al., 2010). Bacteria lacking the capability to colonize and infection can enter plant endophytic niches via wounds and cracks on the plant, which is documented as the passive mode of endophytic colonization (Christina et al., 2013) (**Fig.1**).

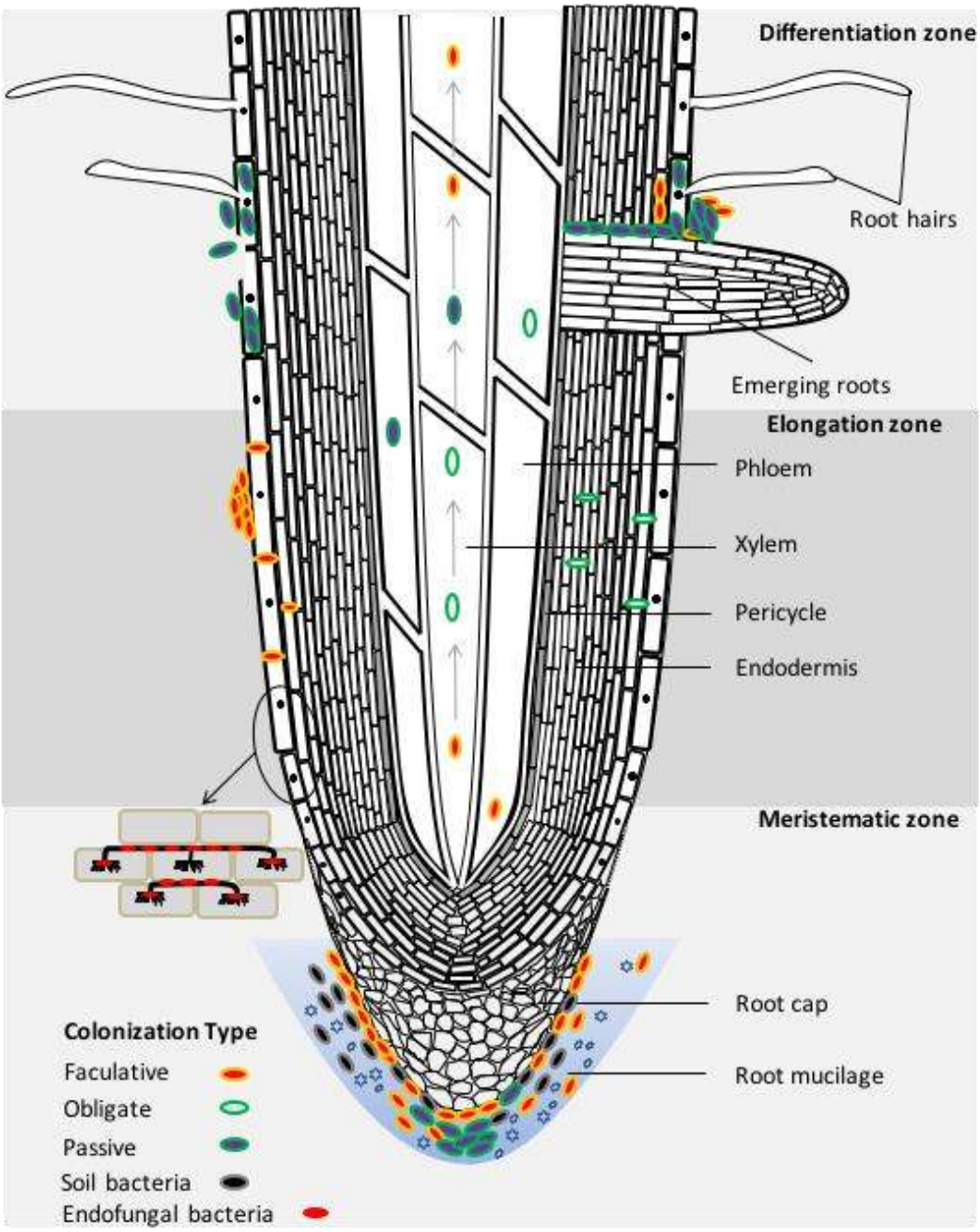

Fig. 1 Schematic representation of the distribution and colonization patterns of endophytic bacteria in the root. Hotspots of colonization sites are the emerging sites of lateral roots and root hairs. Natural wounds and root cracks created by physical damage, root herbivores or nematodes are access points for bacteria to enter roots and translocate to other plant parts by the xylem stream. The arrows show the proposed translocation of bacteria inside the xylem. Endophytic bacteria engaging in different life styles are depicted by colored ovals (not to scale). This illustration was inspired by work conducted by Compant et al. (2005; 2008) and Glaeser et al. (2016).

Current evidence also reveals that some bacteria live in symbiosis with plant endophytic fungi (Desirò et al., 2015; Glaeser et al., 2016). Interestingly, some endofungal bacteria colonize plants in

a similar fashion as their fungal host. For instance, the endofungal bacterium *Rhizobium radiobacter* F4 hosted by the fungus *Piriformospora indica* colonizes plant roots and forms aggregates of attached cells and dense biofilms at the root surface (Glaeser et al., 2016).

Aside from the inner roots belowground, bacteria also widely colonize the endophytic compartments in stem and leaf habitats aboveground (Elbeltagy et al., 2001). **Fig. 2** provides a schematic representation of the colonization patterns of bacteria in a leaf. For instance, the endophytic diazotroph *Herbaspirillum* has been detected in upper epidermis cells, palisade mesophyll cells, xylem vessels as well as spaces between spongy mesophyll layer cells in the leaves of sugarcane plants (Olivares et al., 1997). There is an indication that the endophytic bacteria in aerial plant parts (including leaves) can be translocated from the rhizosphere via plant roots (Lamb et al., 1996), but alternatively, some epiphytes in the phyllosphere enter the interior leaf via the natural openings of stomata, hydathodes, and wounds and cracks generated by insect and pathogen attacks (Vorholt 2012).

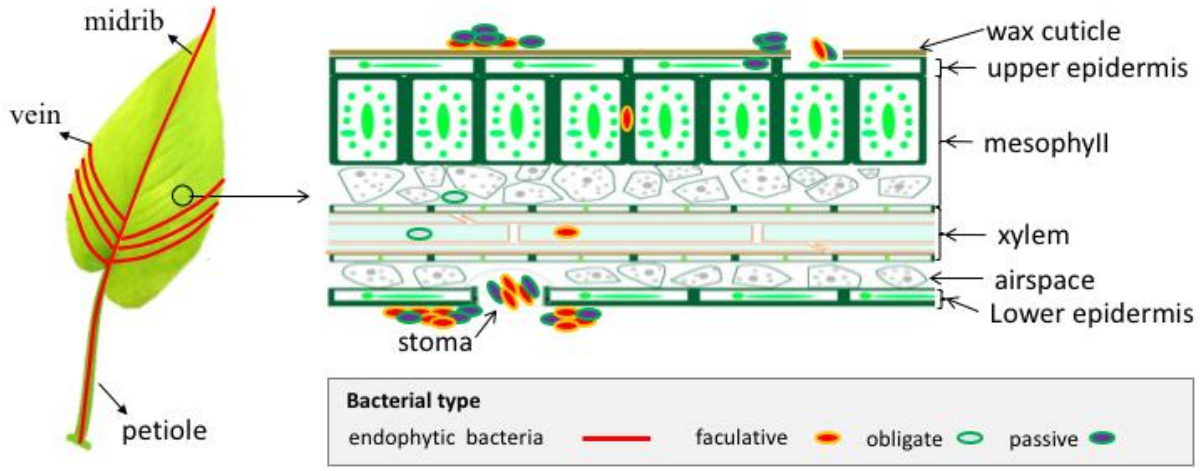

Fig. 2 Schematic representation of colonization patterns of endophytic bacteria in a leaf. Endophytic bacteria can colonize leaf petiole, midrib and veins (as shown in the leaf on the left). Shown on the right is a magnified leaf cross-section. A plant leaf contains arrangements of vascular tissue with xylem and phloem that extend from the leaves through the stem to the roots. Hence root endophytic bacteria are able to translocate to the leaf. Leaf endophytic bacteria may be mainly present in the apoplast but can also colonize intracellularly.

Endophytic bacteria have also been detected in the plant reproductive organs, including flowers, fruits and seeds but normally in very a small number (Compant et al., 2011; Rosenblueth & Martínez-Romero, 2006; Truyens et al., 2015). *Streptomyces mutabilis* strain IA1 isolated from

Saharan soil is able to control the fungal pathogen *Fusarium culmorum* in wheat seedlings and it colonized inside the caryopsis, up to the endocarp layer of wheat (Toumatia et al., 2016). *Pseudomonas* spp. and *Bacillus* spp. were also found to colonize inside the grapevine epidermis and xylem of the ovary or were detected in the intercellular spaces of pulp cells and along cell walls inside seeds when visualized by FISH (Compant et al., 2011).

Overall, it is evidenced that endophytic bacteria colonize both intracellularly and extracellularly in interior of plants. Despite having been detected in all plant parts, roots that have the most intimate contact with soil may function as the first avenue for the recruitment of endophytic bacteria. Endophytic bacteria may have a genetic basis to their different colonization and infection patterns, which may also further correlate to their interaction patterns within plants. In the following sections, we provide more detail on traits that enable endophytic bacteria to successfully establish in plant tissues.

5 Traits for Successful Invasion, Colonization and Translocation of Endophytes

To successfully colonize the inner tissues of plants, endophytic bacteria are equipped with some necessary traits. Motility, chemotaxis, production of cell-wall degrading products and lipopolysaccharide formation are among the observed traits for bacteria to infect and adapt to inside plants (Piromyou et al., 2015). Comparative genomic or metagenomic analyses together with mutational studies have confirmed the importance of these traits. Those genes encoding proteins related to bacterial motility, chemotaxis and adhesion were induced in *B. kururiensis* M130 in the presence of rice plant extracts (Coutinho et al., 2015). This suggests that bacteria may adjust gene expression when infecting and colonizing plants. Adherence to root surface is a crucial step for bacteria to infect plants. Genes encoding Type IV Pili (TFP), the crucial virulence factor formed by pilin subunits, exist in the genome of endophytic bacteria *B. phytofirmans* PsJN (Mitter et al., 2013). Mutant analysis has demonstrated the essential role of TFP-dependent adhesion for the establishment of *Azoarcus* sp. inside rice roots (Dörr et al., 1998). It was further revealed that TFP retraction protein-mediated twitching motility is essential for N_2-fixing bacteria *Azoarus* sp. strain BH72 to establish inside rice roots but this was not important for the colonization on the root surface (Böhm et al., 2007). For beneficial endophytes, the bacterial flagella that typically act as a potent microbe-associated molecular pattern (MAMP) for recognition by the innate immune system may instead mediate endophytic competence by enabling bacterial chemotactic movement and anchoring to plant surfaces (Buschart et al., 2012). The five endophytic bacteria examined by

Straub et al. (2013) all contain the entire flagella machinery and a flagella-deficient mutant was hampered in colonization efficiency of wheat roots (Croes et al., 1993).

Cell-wall degrading enzymes are important for plants to break plant cell walls and translocate compounds to the apoplast. Genes encoding cell-wall degrading enzymes widely exist in the genome of endophytic bacteria (Straub et al., 2013). For example, genes encoding plant-polymer-degrading cellulases, xylanases, cellobiohydrolases, endoglucanase and cellulose-binding proteins were detected in high copy numbers in the metagenome of rice root endophytic bacterial communities (Sessitsch et al., 2012). *In vitro* assays confirmed that endoglucanases are crucial for *Azoarcus* sp. to colonize inside rice roots (Reinhold-Hurek et al., 2006). Endophytic bacteria may also secrete pectinases to degrade the middle lamella between plant cells to ingress intracellularly and translocate within the symplast. For example, pectinase is an important determinant modulating rice early infection by the plant growth promoting bacteria *Bradyrhizobium* sp. SUTN9-2, which originally formed symbiotic relationships with the leguminous weed *Aeschynomene americana* (Piromyou et al., 2015). Pectinesterase expression in this bacterium was up-regulated after being inoculated on rice seedlings (Piromyou et al., 2015). In addition to the abovementioned traits, Kost et al. (2014) found that oxalotrophy, the capacity of utilizing oxalate as a carbon source, is required for the successful colonization of *B. phytofirmans* PsJN on lupin and maize plants. Oxalotrophy was reported to be only associated with plant-beneficial *B. phytofirmans* species, while plant pathogenic or human opportunistic pathogenic species of the *Burkholderia* genus are not able to use oxalate (Kost et al., 2014). This study suggests a role of oxalate in plant selection for beneficial endophytes while avoiding pathogenic bacteria from the complex soil bacterial communities. Overall, the traits discussed above seem to be required for the active invasion and systemic transmission of endophytic bacteria within plants.

6 Bacterial Endophytes Circumvent Host Defense

Plants highly rely on sophisticated defense systems to counteract attacks from phytopathogens (Jones & Dangl, 2006). Microbe-associated molecular pattern (MAMP)-triggered immunity equips plants with a basal level of defense. MAMP-triggered immunity has pattern-recognition receptors as a surveillance system to perceive conserved MAMPs. During the coevolution with plants, pathogens developed the strategy of injecting effectors into plants and interrupt plant MAMP-triggered immunity. In response, plants developed effector-triggered immunity. Within this strategy, plants developed receptors that recognize the effectors of pathogens and then activate a

hypersensitive response leading to programed cell death that also kills the invaders (Jones & Dangl, 2006).

The plant immune systems may also influence bacterial colonization and multiplication inside plants. To avoid antagonistic effects, the endophytic bacteria, unlike phytopathogens, generally do not elicit significant plant immune responses, such as the production of pathogenesis-related (PR) proteins. Many cell surface components of endophytic bacteria are distinct from those of rhizobia and phytopathogens. For example, the flagellin sensing system flg22-Flagellin Sensing 2 (FLS2) in grapevine differentially recognizes the flagellin-derived epitopes of endophytic plant growth promoting bacteria *B. phytofirmans* from those of a bacterial pathogen such as *Pseudomonas aeruginosa* or *Xanthomonas campestris* (Trdá et al., 2014). This difference in plant responses to *B. phytofirmans* and pathogenic bacteria suggest that the flagellin of non-pathogenic endophytic bacteria may have evolved to circumvent recognition of the plant immune system. Other important cell surface components include the bacterial protein secretion systems (SS) which are large protein complexes that transverse the cell envelope and contain a channel mediating the translocation of proteins or protein-DNA complexes (Green & Mecsas, 2016). For Gram-negative and Gram-positive bacteria, eight (Type I SS~ Type VI SS and Sec, and Tat) and six (Sec, Tat, secA2, Sortase, Injectosome and Type VII SS) different protein SS have been described, respectively (Green & Mecsas, 2016; Tseng et al., 2009).

Among the SS, T3SS and T4SS are pivotal for pathogens to deliver effector proteins into plants, which can induce effector-triggered immunity in plants (Green & Mecsas, 2016). However, the endophytic bacteria do not seem to elicit significant plant defense responses, as T3SS and T4SS may be either absent or rare (**Fig.3**). A previous study demonstrated the rare presence of T3SS- and T4SS- encoding genes in the genomes of eleven endophytic bacterial strains via a metagenomic survey (Reinhold-Hurek & Hurek, 2011). Krause et al. (2006) sequenced the whole genome of *Azoarcus* sp. strain BH72 and described it as 'disarmed' due to the lack of both, T3SS and T4SS as well as other important cell surface components usually present in pathogens. It was also demonstrated that the genomic inventory of *Herbaspirillum frisingense* GSF30(T) characterized in biomass grasses lacks T3SS as well as the other four endophytic bacterial strains that were present (*Gluconacetobacter diazotrophicus* PAI5, *Azoarcus* sp. BH72, *Klebsilla pneumoniae* 342, *Azospirillum* sp. B510) (Straub et al., 2013). Additionally, all the endophytic *Herbaspirillum* strains so far examined lack the T4SS that also functions in virulence (Juhas et al., 2008; Straub et al., 2013). However, T3SS and T4SS are crucial for *Bradyrhizobium* sp. SUTN9-2 (isolated from the leguminous grass *Aeschynomene americana* L.) to colonize the roots of rice seedlings (Piromyou et

al., 2015). The function of T6SS is largely unknown but they may also be important for plant-endophytic interactions (Mitter et al., 2013; Sessitsch et al., 2012). In summary, endophytic bacteria tend not to express T3SS and T4SS that induce the plant effector-triggered immunity, but some rhizobium-type endophytic bacteria may require T3SS to colonize the plant endophytic compartment. How the SS of endophytic bacteria interact with plant defense are still not well understood and warrants more studies.

Production of a range of reactive oxygen species (ROS) is typically a non-specific tactic for plant defense (Apel & Hirt, 2004). Colonization of endophytic bacteria also elicits an oxidative burst in rice and the traditional Chinese medicine plant *Atractylodes lancea* (Alquéres et al., 2013; Han et al., 2015; Zhou et al., 2015). To detoxify the initial ROS produced by the plant, the endophytic bacteria may resort to ROS scavenging enzymes for help (**Fig.3**). A high number and diversity of genes encoding enzymes involved in ROS scavenging, such as superoxide dismutase (SOD) and glutathione reductase (GR) are represented in the metagenome of the endophytic bacterial communities in rice roots (Sessitsch et al., 2012). Genes encoding enzymes involved in ROS scavenging were also detected in the genome of *Enterobacter* sp. 638 (Taghavi et al., 2010). ROS-scavenging enzymes are reported to be involved in the biological N fixation process of *Gluconacetobacter diazotrophicus* and are essential for its successful colonization in endophytic rice roots (Alqueres et al., 2010; 2013). The transcript levels of ROS-scavenging enzyme-encoding genes were upregulated in *G. diazotrophicus* strain PALS when they colonized the plant interior.

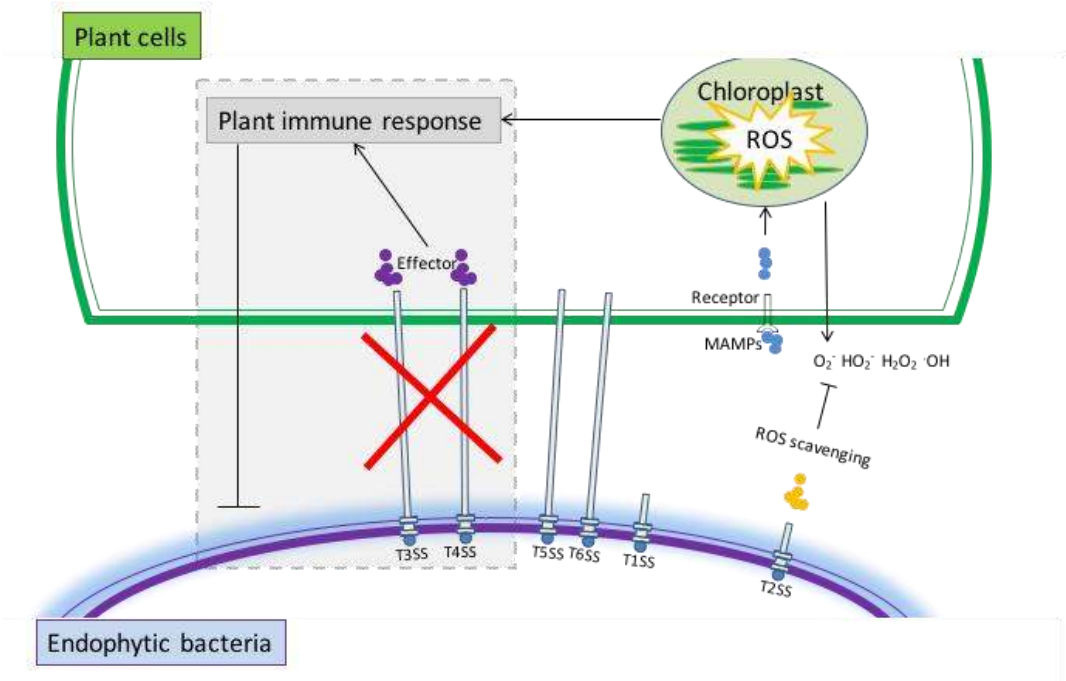

Fig. 3 Schematic representation summarizing the typical properties of endophytic bacteria to cope with plant defenses. The lack of T3SS and T4SS of endophytic bacteria does not cause significant plant immune response, which may contribute to the successful colonization of bacterial inside plants. Information for this illustration was sourced from Alqueres et al (2013), Reinhold-Hurek and Hurek (2011), and Straub et al (2013).

Avoiding excessive growth is another mechanism for bacteria to establish in plant tissues without causing plant defense responses. The endophytic bacterium *Xylella fastidiosa* is a plant disease-causing agent. A study using an *rpfF*-deficient mutant found that cell-to-cell signaling mediated by a fatty acid diffusible signaling factor is central to the regulation of *X. fastidoisa* virulence as it restricts the movement and colonization by *X. fastidiosa* within grapevine plants. The *rpfF* gene led to a reduced virulence of *X. fastidiosa* on grapes (Chatterjee et al., 2008). In summary, endophytic bacteria can employ a range of strategies to cope with the antagonistic effects of plant defenses.

7 Plant Hormone Signaling Pathways and Endophytic Bacterial Colonization

Several studies have investigated how plant defense signaling regulates the colonization of bacteria inside plants. The activation of the ethylene (ET) signaling pathway suppressed the endophytic colonization of *Medicago truncatula* by the plant growth promoting bacterium *Klebsiella pneumoniae* 342 (Kp342) and the human enteric pathogen *Salmonella enterica* serovar Typhimurium (Iniguez et al., 2005). Furthermore, an ET-insensitive *M. truncatula* mutant was hypercolonized by Kp342 compared with the wild-type plants (Iniguez et al., 2005). In line with this study, it was found that the activation of jasmonate (JA) signaling suppressed rice root colonization by *Azoarcus* sp. strain BH72 (Miché et al., 2006). Additionally, the activation of JA signaling also strongly suppressed early stage nodulation in *Lotus japonicus* (Nakagawa & Kawaguchi, 2006). These studies indicate that enhanced plant signaling may restrict the colonization of specific endophytic bacteria or rhizobium in plant endophytic environments.

The diversity of endophytic bacterial communities may influence plant defense capabilities. This perception is supported by the observation that inside roots of wilt (caused by *Ralstonia solanacearum*) resistant tomato cultivar *Arka Abha*, bacterial diversity was higher than in the susceptible cultivar *Arka Vikas* (Upreti & Thomas, 2015). Moreover, the wilt-resistant cultivar harbored bacteria that were more likely to employ antimicrobial strategies (production of

siderophores and HCN) than the wilt-susceptible cultivar (Upreti & Thomas, 2015). Therefore, it may bear significant importance to investigate how plant defense signaling pathways interact with the diversity and composition of endophytic bacterial communities. The diversity of endophytic bacterial communities in *Arabidopsis* leaves decreased by the activation of salicylic acid (SA) signaling, but the communities were not influenced by the activation of the JA-dependent defense pathway (Kniskern et al., 2007). Conclusions of this study were based on the abundance of 30 culturable bacterial groups, which may be a low sample size for major conclusions to be based on. A recent study by Lebeis et al. (2015) provides evidence that plant roots sculpt their endophytic bacterial communities differentlly in different isogenic *Arabidopsis* defense signaling mutants. However, this was observed at the family level but not at a genus/species level. ET signaling influenced the endophytic bacterial communities in *Nicotiana attenuate*. It was observed that isogenic transformed plants impaired in ET biosynthesis (*ir-aco1*) or perception (*35S-etr1*) plants harbored culturable bacterial communities of a lower diversity than wild-type plants (Long et al., 2010). From the abovementioned studies, it seems that contrary to the studies on single bacterial isolates, the influence of the signaling pathways on endophytic bacterial communities are variable and may be small.

In summary, plant defense pathways may influence the diversity of endophytic bacteria through restricting colonization routes (**Fig. 4**). The suppression effect is ecologically logical as the plant innate immune system should help plants to control the 'over-presence' of any hosted bacteria and to maintain an optimum bacterial density inside plants. The application of plant hormones might also be used for the manipulation of plant endophytic bacteria, e.g. to control human pathogens present in food, such as *Salmonella* strains inside vegetables (Iniguez et al., 2005).

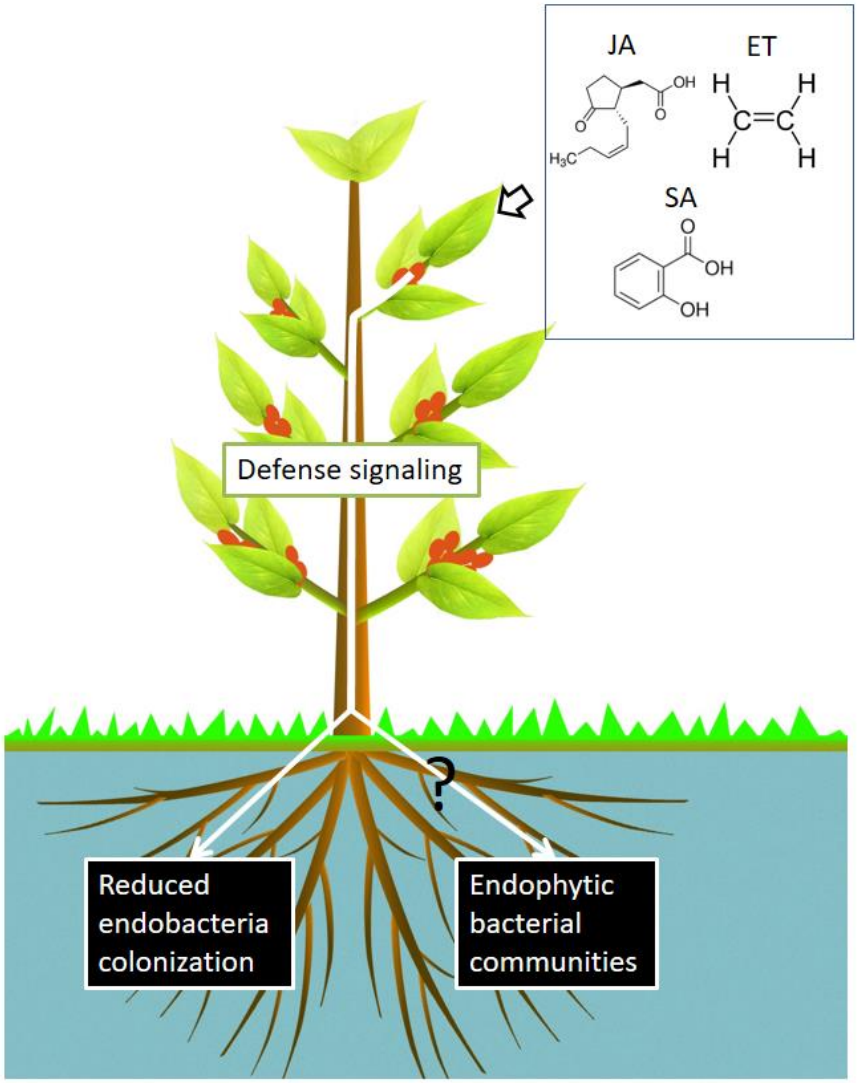

Fig. 4 Elevated plant SA, JA and ET signaling obtained by exogenously applied phytohormone treatment suppresses bacterial colonization in the plant endophytic root environment, but the mechanisms underlying this selective colonization by endophytic bacterial communities are currently not understood (Kniskern et al. 2007; Lebeis et al. 2015; Miché et al. 2006; Iniguez et al. 2005).

8 Plant Growth-Promoting Traits

It is expected that elucidation of the processes involved in plant growth promoting traits (PGPTs) will facilitate the development of potent biofertilizers and promote a sustainable agriculture (**Fig. 5**). Endophytic bacterial communities and even a single endophytic bacterial strain can have multiple PGPTs (Miliute et al., 2016; Rolli et al., 2015; Tsurumaru et al., 2015). Generally, growth

stimulation by endophytic bacteria can be a consequence of phytohormone production, elicitation of plant priming conditions, suppression of phytopathogens and/or improvement of plant nutrition (**Fig. 6**). These PGPTs of endophytic bacteria are briefly discussed with an agricultural focus in the following sections.

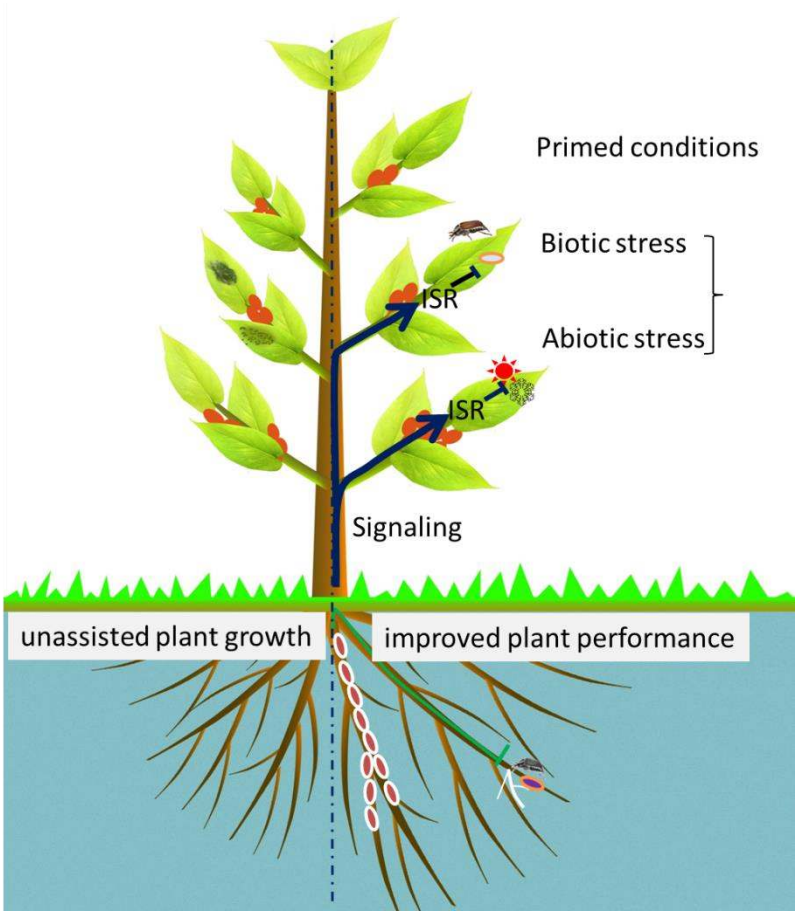

Fig. 5 Visualization of endophytic bacteria-induced systemic resistance (ISR). The right half of this illustration presents the elicitation of plant primed conditions by endophytic bacteria. Some beneficial effects may include changes in root architecture relative to the uninoculated plants as shown on the left. The endophytic bacteria-mediated ISR may be modulated by either one or combined signaling cascades of SA, JA and ET in an endophytic bacteria-dependent manner.

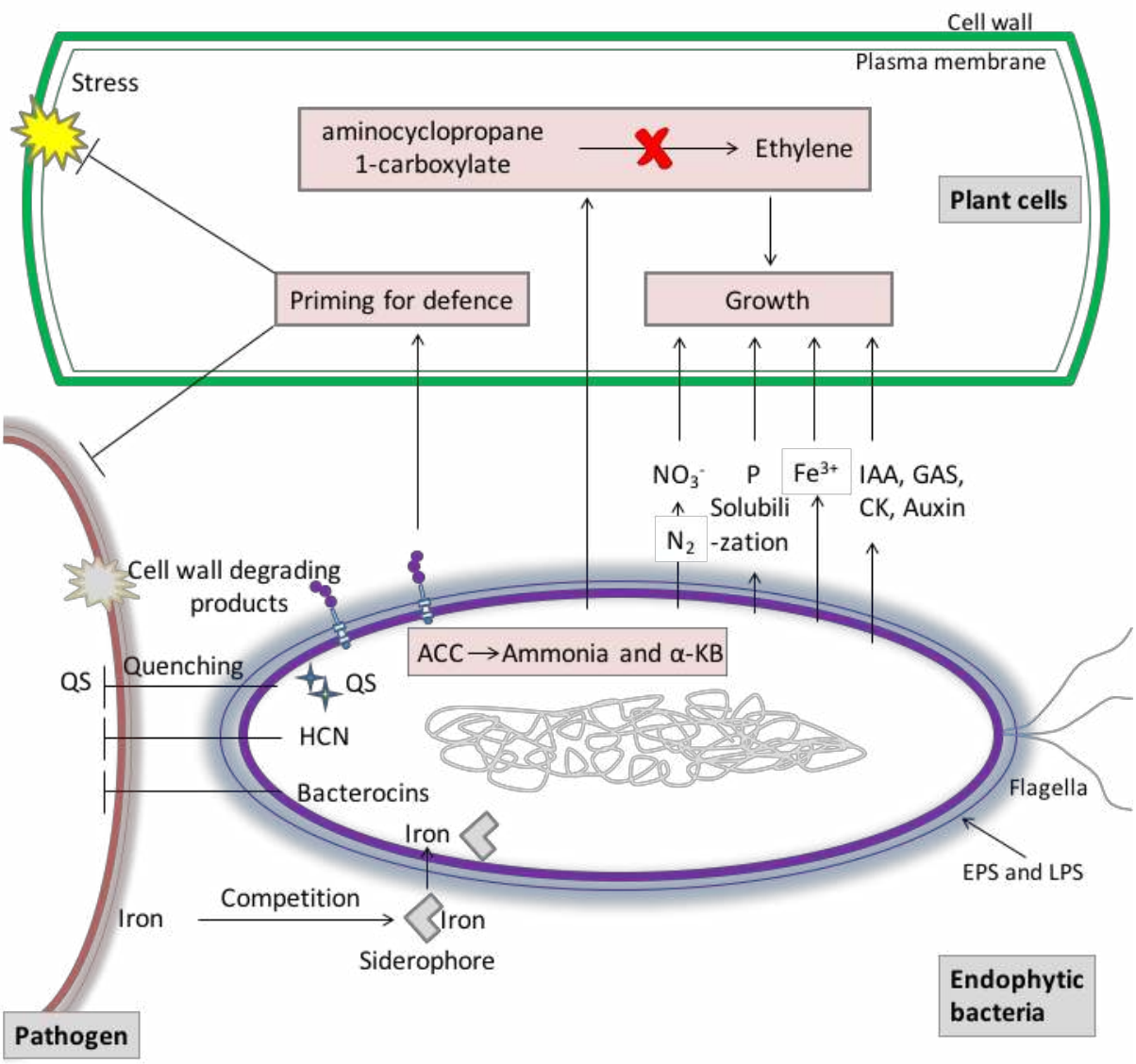

Fig. 6 Schematic representation summarizing proposed plant growth promoting traits (PGPTs) of endophytic bacteria. Endophytic bacteria may promote plant biomass production by providing solubilizing phosphate, assimilable N to plants and by suppressing ethylene synthesis in plant. The indirect plant growth promoting effects are mainly related to biocontrol, especially in the plant root areas, that are mediated by the production of antimicrobial agents and siderophores, competition for nutrients, and the induction of plant defense. The arrows denote the plant-endophytic bacteria interactions and the symbol '⊥' indicates inhibition. Abbreviations: QS, quorum sensing; IAA, indoleacetic acid; ACC, 1 aminocyclopropane-1-carboxylic acid; GAs, the gibberellins; CK, cytokinin, EPS, extracellular polymeric substance; LPS, lipopolysaccharide; αkb, α-ketobutyrate.

8.1 Phytohormone Production

Producing phytohormones is a common feature of endophytic bacteria to promote plant growth, and increase stress tolerance (Pieterse et al., 2009). IAA (Zúñiga et al., 2013), cytokinins (CKs) (Bhore et al., 2010) and GAs (Shahzad et al., 2016) are phytohormones that can be synthesized by endophytic bacteria. Genes encoding proteins involved in biosynthesis of phytohormones are often present in the metagenome of plant endophytic bacterial communities; e.g., four pathways of IAA biosynthesis were detected in the metagenome of the tomato root gall-associated microbiome (Tian et al., 2015). Inoculation with endophytic bacteria may benefit plants via the production of phytohormones. For instance, the endophytic bacterium *Sphingomonas sp.* LK11 enhanced tomato growth, which may have been mediated by the production of GAs and IAA (Khan et al., 2014). Another interesting study showed that *Luteibacter* sp. promoted the IAA production by its fungal host, the foliar fungal endophyte *Pestalotiopsis* aff. neglecta (Hoffman et al., 2013). This highlights that there are many important indirect plant microbial interactions that promote plant growth that are rarely considered. Additionally, *S. mutabilis* strain IA1 isolated from a Saharan soil was able to produce IAA and GA3 and inoculation of this bacterium on wheat seedlings reduced the progression and severity of *F. culmorum* infection (Toumatia et al., 2016). Evidently enhancing phytohormone production using endophytic bacteria for increased crop production in agriculture is promising.

8.2 Suppression of Phytopathogens

8.2.1 Endophytic Bacterial Colonization Primes Plants for Enhanced Defense

Plant priming conditions are commonly seen in nature (Conrath et al., 2015). Exogenous treatment with a low dose of JA, SA or ET as well as infections by less aggressive phytopathogens or herbivores may trigger this primed state (Conrath et al., 2015). Such defense priming can also be induced by plant-beneficial bacteria interactions (Pieterse et al., 2014). JA, SA or ET signaling pathways are generally involved in this process. An increasing number of studies have demonstrated this capability of endophytic bacteria. For instance, *Azoarcus* sp. strain BH72 induced a JA-dependent pathway response in rice roots in a cultivar-dependent manner, triggering more responses in the less compatible cultivar IR42 than in the compatible cultivar IR36 (Miché et al., 2006). Specifically, the protein PR10 was induced in IR42 by the endophytic colonization by this *Azoarcus* sp. strain (Miché et al., 2006). Another study pointed at *E. radicincitans* DSM 16656 as a highly competitive colonizer in the endophytic environment of various agricultural vegetables and crops (Brock et al., 2013). Transcriptional profiling of the defense-related PR genes *PR1*, *PR2*, *PR5*

and *PDF1.2* revealed that this bacterium is able to switch *Arabidopsis* to the primed state via SA- and JA/ET- dependent pathways (Brock et al., 2013). Some bacterial strains are also able to live in symbiosis with plant endophytic fungi (Desirò et al., 2015; Glaeser et al., 2016). It was recently discovered that the endofungal bacterium *Rhizobium radiobacter* F4, like its fungal host *P. indica*, increased plant resistance against the bacterial leaf pathogen *Pseudomonas syringae* pv. tomato DC3000. Mutational analysis of this strain confirmed that this resistance was mediated by a JA-dependent pathway (Glaeser et al., 2016).

8.2.2 Interruption of the Signaling of Phytophathogens

Quorum sensing (QS) is a crucial strategy for bacteria to survive in complex ecological niches. It regulates the physiological activities of bacteria, involving cell-to-cell communication, reproduction, biofilm formation, competence and adaptations (Miller & Bassler, 2001). Certain endophytic bacteria employ QS quenching as an antivirulence strategy to control phytopathogens. For instance, certain endophytic bacterial strains in *Cannabis sativa* L. disrupt the cell-to-cell communication of the biosensor strain *Chromobacterium violaceum* via quenching its QS signals (Kusari et al., 2014). A similar mechanism could be deployed in an agricultural context. For example, diffusible signal factor (DSF) is necessary for the virulence of several *Xanthomonas* species and *Xylella fastidiosa* (Newman et al., 2008). Thereof, *Bacillus* and *Pseudomonas* complemented with *carAB*, a gene required for the fast DSF degradation in *Pseudomonas* spp. strain G, can possibly be used to biocontrol these DSF producing pathogens. This strategy of attenuating the virulence of phytopathogens via interruption of QS of phytopathogens may provide a new paradigm of developing biocontrol agents for sustainable agriculture.

8.3 Biocontrol by Endophytic Bacteria

As endophytic bacteria colonize similar locations as pathogenic bacteria inside plants, this may provide opportunities for their use as biocontrol agents. The antimicrobial compounds produced by endophytic bacteria are promising in providing sufficient protection for plants against the invasion of phytopathogens. For instance, *Bacillus amyloliquefaciens* 41B-1 (an endophytic bacterium isolated from cotton) exhibited strong suppression of cotton wilt disease (caused by *Verticillium dahlia*) under glasshouse conditions (Han et al., 2015). A series of isoforms of iturins in *B. amyloliquefaciens* may be responsible for the observed antifungal effects. Exogenous treatment with the purified iturins triggered a ROS burst, cell-wall disintegrity and affected the fungal

signaling pathways (Han et al., 2015). Apart from these antagonisms, iturins also acted as activators to induce the MAMPs-triggered immunity defense in cotton plants (Han et al., 2015). Therefore, iturins are promising antimicrobial compounds for use in future plant protection.

Endophytic bacteria can also increase plant pathogen resistance by producing volatile organic compounds (VOCs) (Chung et al., 2016). *E. aerogenes*, an endophytic bacterium that colonizes maize plants, is one of the main producers of the VOC 2,3-butanediol (2,3-BD) (D'Alessandro et al., 2014). Maize plants inoculated by this bacterium showed increased resistance towards the Northern corn leaf blight (caused by the fungus *Setosphaeria turcica*). However, the inoculated plants were more vulnerable to the caterpillar *Spodoptera littoralis*.

A trait that is known for enabling endophytic bacteria to compete with other pathogenic bacteria for available iron is siderophore-production (Compant et al., 2005a). For instance, *Bacillus subtilis* GY-IVI improved plant growth of muskmelon (*Cucumis melo* L.) potentially by increasing the production of siderophores, IAA and ammonia (Zhao et al., 2011). In another experiment, *Azadirachta indica* A. Juss seeds were inoculated with a spore suspension containing three *Streptomyces* endophytic strains AzR-051, AzR-049 and AzR-010 from *A. indica* root tissues (Verma et al., 2011). The inoculated seeds displayed significantly higher plant growth and inhibition of the fungal pathogen *Alternaria alternata*, which causes early blight disease in tomato plants. It has been hypothesized that this observation results from the production of siderophores (Verma et al., 2011). These studies provide compelling evidence that siderophores produced by endophytic bacteria have vital roles in inhibiting the progressiveness of phytopathogens (Verma et al., 2011). In another example, the endophytic *P. poae* strain RE*1-1-14 that was originally isolated from sugar beet roots, was able to suppress the fungal pathogen *Rhizoctonia solani* (Zachow et al., 2015). A novel lipopeptide poaeamide produced by this bacterium may relate to its suppression towards *R. solani* and its establishment in sugar beet roots. Despite the potential scope and impact that these biocontrol traits could have in agriculture, their application in field conditions is still in its infancy.

8.4 Abiotic Stress

The mechanisms underlying the endophytic bacteria-mediated improvements of plant resistance to abiotic stress are starting to be elucidated. For example, psychrotolerant endophytic bacteria *P. vancouverensis* OB155 and *P. frederiksbergensis* OS261 were found to be able to protect tomato

plants from cold stress (10-12°C) (Subramanian et al., 2015). Relative to the control tomato plants, less membrane damage and ROS production, and increased antioxidant activity were observed in the leaves of bacteria-inoculated plants. Meanwhile, an induced expression of cold acclimation genes (*LeCBF1* and *LeCBF3*) was detected in both bacteria-inoculated and cold stress-treated plants. Similarly, inoculation of the beneficial endophytic *B. phytofirmans* strain PsJN contributed to cold stress resistance in *Arabidopsis* (Su et al., 2015). An increased plant growth and a strengthened cell wall were also observed upon bacterial inoculation (Su et al., 2015).

Endophytic bacteria have also been reported to increase plant tolerance to drought. Transcriptome analysis showed that endophyte *B. phytofirmans* PsJN-inoculated potato plants displayed a diverse range of functionalities (Sheibani-Tezerji et al., 2015). Transcripts involved in transcriptional regulation, cellular homeostasis and ROS detoxification were upregulated in *B. phytofirmans* PsJN in drought stress-affected potato. This suggests that endophytes can sense physiological changes in plants and adjust the expression of a set of genes. In summary, endophytic bacteria therefore have the potential to be used as protective agents in agriculture systems to extreme climatic environments via alterations of plant physiological conditions.

8.5 Endophytic Bacteria Promote Plant Nutrient Uptake

Siderophore Production Iron is essential for all living organisms but the bioavailable iron in soil is limited. The production of siderophores can increase plant growth by chelating iron in the soil and producing soluble complexes that can be absorbed by several plants. Alternatively, siderophores deprive phytopathogens of iron by binding to the bioavailable forms of iron first (Aznar et al., 2015; Verma et al., 2011). Siderophore production may assist plants' establishment in a soil of low iron conditions. In a previous study we demonstrated that plants lacking soil bacteria suffer from iron deficiency (Carvalhais et al., 2013). Genes encoding proteins that function in siderophore biosynthesis, siderophore reception and iron storage have been detected in large copies in rice root endophyte communities, which suggests a great potential of the rice root microbiomes for assisting with rice iron uptake (Sessitsch et al., 2012). In another example, the key role of siderophore production of endophytic *Streptomyces* sp. GMKU 3100 in its beneficial process towards rice plant has been established via studying a siderophore-deficient-mutant (Rungin et al., 2012).

Nitrogen Metabolism Available N is crucial for plant growth and health. Approximately 30~50% of the N in crop field is from biological fixation of N_2 by soil microorganisms, including from free-

living diazotrophs (Gourion et al., 2015). Some endophytic bacteria possess both the nitrogen fixation genes of *nifH* and denitrification genes (Straub et al., 2013). Endophytic bacterial communities can potentially affect the nitrification and ammonia oxidation in rice roots, as a considerable number of genes involved in N cycling were detected in the metagenome of the endophytic rice root microbiome (Sessitsch et al., 2012). *Paenibacillus polymyxa* P2b-2R isolated from lodgepole pine tissue was found to colonize the rhizosphere and endophytic compartment and is able to fix N_2 and promote the growth of corn (Puri et al., 2016). The importance of endophytic bacteria in plant N cycling is also supported by evidence that N_2 fixation by foliar endophytic bacteria has occurred in many subalpine conifer species (Moyes et al., 2016). N_2-fixing by endophytes may provide long-lived conifers with a low-cost and evolutionarily stable way for N nutrient supply.

8.6 1-aminocyclopropane-1-carboxylate (ACC) Deaminase

ET is produced in plants during stress responses leading to decreased plant growth or even death if present in high concentrations (Glick et al., 2014). Some bacteria, including endophytes, use the precursor of ethylene (1-aminocyclopropane-1-carboxylate - ACC) as a carbon source through the production of an enzyme known as ACC deaminase (Glick et al., 2014; Ali et al., 2014; Karthikeyan et al., 2012; Zhang et al., 2011). Plants exposed to abiotic stress (e.g., flooding, drought, salinity) accumulate ACC in roots, which can systematically spread to shoots and leaves where it is converted to ET (Tudela & Primo-Millo, 1992). Inoculation with bacterial ACC deaminase producers may endow plant tolerance to various abiotic stresses (Glick et al., 2014). A recent study revealed 13 endophytic bacterial isolates containing efficient ACC deaminase activity from the inner tissues of halophytic *Limonium sinese* (Girard) (Qin et al., 2014). Four strains were selected based on their improvement towards seed germination of *L. sinese* seedlings under different salt stress concentrations. Significantly higher root length, shoot length, leaf area and leaf numbers were observed upon inoculation. All four strains were saline-tolerant when exposed to 7% (w/v) NaCl concentrations (Qin et al., 2014). This study supports that ACC deaminase activity assists plants to tolerate abiotic conditions by lowering ET levels. The isolation of habitat-adapted endophytic bacteria may potentially identify more strains with ACC deaminase activity to provide new benefits to numerous plants.

9 Concluding Remarks and Prospects

Phylogenetic marker gene sequencing surveys and meta'omic analyses have greatly increased our knowledge of endophytic bacterial microbiomes. It seems like plants sculpt their root endophytic microbiome to support their growth and defense and roots act as effective gatekeepers in this process. However, it still remains unclear how bacteria in the leaf endophytic compartments contribute significantly to plant health and growth. Typically, microbial communities in plant endophytic environments are of low diversity, and are dominated by Proteobacteria and Actinobacteria. Coincidentally, these are also the bacterial phyla that harbor the highest numbers of plant growth promoters. Many of the plant growth promoting traits of endophytic bacteria suggest that they are good candidates for the development of biofertilizers in agriculture.

Endophytic bacteria, as a result of co-evolution, are highly adapted to the environment inside plant tissues, and elucidation of the mechanisms behind these dynamic populations despite effective plant defense mechanisms still warrants future studies. It will be of great interest in the future to decode why Acidobacteria and Archaea are not competent colonizers in plant endophytic compartments and to reveal the relevant ecological reason behind this phenomenon. A descriptive picture of the endophytic bacterial community composition may be able to predicate potential functions of these microbiomes. Phylogeny complemented with function-based approaches to study endophytic bacterial communities should be helpful in revealing microbial determinants influencing plant health and yields.

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Declaration of Interest Statement

The authors declare no conflict of interest.

References

Ali S, Charles TC, Glick BR. (2014). Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. Plant Physiol Bioch 80:160-167.

Alquéres S, Meneses C, Rouws L, et al. (2013). The Bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by *Gluconacetobacter diazotrophicus* PAL5. Mol Plant Microbe In 26:937-945.

Alquéres SMC, Oliveira JHM, Nogueira EM, et al. (2010). Antioxidant pathways are up-regulated during biological nitrogen fixation to prevent ROS-induced nitrogenase inhibition in *Gluconacetobacter diazotrophicus*. Arch Microbiol 192:835-841.

Anand R, Chanway CP. (2013). Detection of GFP-labeled *Paenibacillus polymyxa* in autofluorescing pine seedling tissues. Biol Fert Soils 49:111-118.

Anand R, Grayston S, Chanway C. (2013). N_2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa*. Microb Ecol 66:369-374.

Apel K, Hirt H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373-399.

Aznar A, Chen NW, Thomine S, Dellagi A. (2015). Immunity to plant pathogens and iron homeostasis. Plant Sci 240: 90-97.

Bai Y, Müller DB, Srinivas G, et al. (2015). Functional overlap of the *Arabidopsis* leaf and root microbiota. Nature 528:364-369.

Beltran-Garcia MJ, White JF, Prado FM, et al. (2014). Nitrogen acquisition in *Agave tequilana* from degradation of endophytic bacteria. Sci Rep 4:6938

Bhore SJ, Nithya R, Loh CY, Kangueane p. (2010). Screening of endophytic bacteria isolated from leaves of Sambung Nyawa [*Gynura procumbens* (Lour.) Merr.] for cytokinin-like compounds. Bioinformation 5:191-197.

Bodenhausen N, Horton MW, Bergelson J. (2013). Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PLoS One 8:e56329.

Böhm M, Hurek T, Reinhold-Hurek B. (2007). Twitching motility is essential for endophytic rice colonization by the N_2-fixing endophyte *Azoarcus* sp. Strain BH72. Mol Plant Microbe In 20:526-533.

Brock AK, Berger B, Mewis I, Ruppel S. (2013). Impact of the PGPB *Enterobacter radicincitans* DSM 16656 on growth, glucosinolate profile, and immune responses of *Arabidopsis thaliana*. Microb Ecol 65:661-670.

Bulgarelli D, Rott M, Schlaeppi K, et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488:91-95.

Bulgarelli D, Schlaeppi K, Spaepen S, et al. (2013). Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807-838.

Bulgari D, Casati P, Quaglino F, Bianco PA. (2014). Endophytic bacterial community of grapevine leaves influenced by sampling date and phytoplasma infection process. BMC Microbiol 14:1-11.

Buschart A, Sachs S, Chen X, et al. (2012). Flagella mediate endophytic competence rather than act as MAMPs in rice-*Azoarcus* sp. strain BH72 interactions. Mol Plant Microbe In 25:191-199.

Carrell AA, Frank AC. (2015). Bacterial endophyte communities in the foliage of coast redwood and giant sequoia. Front Microbiol 6:1008.

Carvalhais LC, Muzzi F, Tan CH, et al. (2013) Plant growth in *Arabidopsis* is assisted by compost-soil derived microbial communities. Front Plant Sci 4:235.

Chatterjee S, Newman KL, Lindow SE. (2008). Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. Mol Plant Microbe In 21:1309-1315.

Chi F, Shen SH, Cheng HP, et al. (2005). Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Appl Environ Microb 71:7271-7278.

Christina A, Christapher V, Bhore SJ. (2013). Endophytic bacteria as a source of novel antibiotics: an overview. Phcog Rev 7:11-16.

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.

Compant S, Clément C, Sessitsch A. (2010). Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42: 669-678.

Compant S, Duffy B, Nowak J, et al. (2005a). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microb 71:4951-4959.

Compant S, Kaplan H, Sessitsch A, et al. (2008). Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. FEMS Microbiol Ecol 63:84-93.

Compant S, Mitter B, Colli-Mull JG, et al. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microb Ecol 62:188-197.

Compant S, Reiter B, Sessitsch A, et al. (2005b). Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl Environ Microb 71:1685-1693.

Conrath U, Beckers GJ, Langenbach CJ, Jaskiewicz MR. (2015). Priming for enhanced defense. Annu Rev Phytopathol 53:97-119.

Coutinho BG, Licastro D, Mendonça-Previato L, et al. (2015). Plant-influenced gene expression in the rice endophyte *Burkholderia kururiensis* M130. Mol Plant Microbe In 28:10-21.

Croes CL, Moens S, van Bastelaere E, et al. (1993). The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. Microbiology. 139:2261-2269.

D'Alessandro M, Erb M, Ton J, et al. (2014). Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions. Plant Cell Environ 37:813-826.

de Almeida Lopes KB, Carpentieri-Pipolo V, Oro TH, et al. (2016). Culturable endophytic bacterial communities associated with field-grown soybean. J Appl Microbiol 120:740-755.

de Oliveira Costa LE, de Queiroz MV, Borges AC, et al. (2012). Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (*Phaseolus vulgaris*). Braz J Microbiol 43:1562-1575.

Deng ZS, Zhao LF, Kong ZY, et al. (2011). Diversity of endophytic bacteria within nodules of the *Sphaerophysa salsula* in different regions of Loess Plateau in China. FEMS Microbiol Ecol 76:463-475.

Desirò A, Faccio A, Kaech A, et al. (2015). *Endogone*, one of the oldest plant-associated fungi, host unique Mollicutes-related endobacteria. New Phytol 205:1464-1472.

Ding T, Melcher U. (2016). Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. PloS One 11:e0150895.

Dörr J, Hurek T, Reinhold-Hurek B. (1998). Type IV pili are involved in plant-microbe and fungus-microbe interactions. Mol Microbiol 30:7-17.

Edwards J, Johnson C, Santos-Medellín C, et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. P Natl Acad Sci USA 112:E911-E920.

Elbeltagy A, Nishioka K, Sato T, et al. (2001). Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl Environ Microb 67:5285-5293.

Ferrando L, Scavino AF. (2015). Strong shift in the diazotrophic endophytic bacterial community inhabiting rice (*Oryza sativa*) plants after flooding. FEMS Microbiol Ecol 91:fiv104.

Fisher P, Petrini O, Scott HL. (1992). The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). New Phytol 122:299-305.

Frommel MI, Nowak J, Lazarovits G. (1991). Growth enhancement and developmental modifications of *in vitro* grown potato (*Solanum tuberosum* spp. tuberosum) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiol 96:928-936.

Glaeser SP, Imani J, Alabid I, et al. (2016). Non-pathogenic *Rhizobium radiobacter* F4 deploys plant beneficial activity independent of its host *Piriformospora indica*. ISME J 10:871-884.

Glick, B.R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169: 30-39.

Gourion B, Berrabah F, Ratet P, Stacey G. (2015). Rhizobium–legume symbioses: the crucial role of plant immunity. Tr Plant Sci 20:186-194.

Green ER, Mecsas J. (2016). Bacterial secretion systems–An overview. Microbiol Spetr 4(1): doi:10.1128/microbiolspec

Hameed A, Yeh MW, Hsieh YT, et al. (2015). Diversity and functional characterization of bacterial endophytes dwelling in various rice (*Oryza sativa* L.) tissues, and their seed-borne dissemination into rhizosphere under gnotobiotic P-stress. Plant Soil 394:177-197.

Han Q, Wu F, Wang X, et al. (2015). The bacterial lipopeptide iturins induce *Verticillium dahliae* cell death by affecting fungal signalling pathways and mediate plant defence responses involved in pathogen-associated molecular pattern-triggered immunity. Environ Microbiol 17:1166-1188.

Hardoim PR, Nazir R, Sessitsch A, et al. (2013). The new species *Enterobacter oryziphilus* sp nov and *Enterobacter oryzendophyticus* sp nov are key inhabitants of the endosphere of rice. BMC Microbiol 13:164

Hardoim PR, van Overbeek LS, van Elsas JD. (2008). Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463-471

Hoffman MT, Gunatilaka MK, Wijeratne K, et al. (2013). Endohyphal bacterium enhances production of indole-3-acetic acid by a foliar fungal endophyte. PloS One 8:e73132.

Hurek T, Reinhold-Hurek B. (2003). *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. J Bio Technol 106:169-178.

Iniguez AL, Dong Y, Carter HD, et al. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. Mol Plant Microbe In 18:169-178.

Jones JDG, Dangl JL. (2006). The plant immune system. Nature 444:323-329.

Juhas M, Crook DW, Hood DW. (2008). Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. Cell Microbiol 10:2377-2386.

Karthikeyan B, Joe MM, Islam MR, et al. (2012). ACC deaminase containing diazotrophic endophytic bacteria ameliorate salt stress in *Catharanthus roseus* through reduced ethylene levels and induction of antioxidative defense systems. Symbiosis 56:77-86.

Khan AL, Waqas M, Kang SM, et al. (2014). Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. J Microbiol 52:689-695.

Kniskern JM, Traw MB, Bergelson J. (2007). Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on *Arabidopsis thaliana*. Mol Plant Microbe In 20:1512-1522.

Koiv V, Roosaare M, Vedler E, et al. (2015). Microbial population dynamics in response to *Pectobacterium atrosepticum* infection in potato tubers. Sci Rep 5:11606.

Koskimäki JJ, Pirttilä AM, Ihantola E-L, et al. (2015). The intracellular scots pine shoot symbiont *Methylobacterium extorquens* DSM13060 aggregates around the host nucleus and encodes eukaryote-like proteins. MBio 6:e00039-00015.

Kost T, Stopnisek N, Agnoli K, et al. (2014). Oxalotrophy, a widespread trait of plant-associated Burkholderia species, is involved in successful root colonization of lupin and maize by *Burkholderia phytofirmans*. Front Microbiol 4:421.

Krause A, Ramakumar A, Bartels D, et al. (2006). Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus* sp. strain BH72. Nature Bio Technol 24.

Kusari P, Kusari S, Lamshöft M, et al. (2014). Quorum quenching is an antivirulence strategy employed by endophytic bacteria. Appl Microbiol Bio T 98:7173-7183.

Lamb TG, Tonkyn DW, Kluepfel DA. (1996). Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. Can J Microbiol 42:1112-1120.

Lebeis SL, Paredes SH, Lundberg DS, et al. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science 349:860-864.

Long HH, Sonntag DG, Schmidt DD, Baldwin IT. (2010). The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. New Phytol 185:554-567.

Lundberg DS, Lebeis SL, Paredes SH, et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. Nature 488:86-90.

Magnani G, Cruz L, Weber H, et al. (2013). Culture-independent analysis of endophytic bacterial communities associated with Brazilian sugarcane. Genet Mol Res 12:4549-4558.

Marques JM, da Silva TF, Vollu RE, et al. (2015). Bacterial endophytes of sweet potato tuberous roots affected by the plant genotype and growth stage. Appl Soil Ecol 96:273-281.

Mendes R, Kruijt M, de Bruijn I, et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332: 1097-1100.

Miché L, Battistoni F, Gemmer S, et al. (2006). Upregulation of jasmonate-inducible defense proteins and differential colonization of roots of *Oryza sativa* cultivars with the endophyte *Azoarcus* sp. Mol Plant Microbe In 19:502-511.

Miliute I, Buzaite O, Gelvonauskiene D, et al. (2016). Plant growth promoting and antagonistic properties of endophytic bacteria isolated from domestic apple. Zemdirbyste 103:77-82.

Miller MB, Bassler BL. (2001). Quorum sensing in bacteria. Annu Rev Microbiol 55:165-199.

Mitter B, Petric A, Shin MW, et al. (2013). Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Front Plant Sci.4:120.

Mora-Ruiz MdR, Font-Verdera F, Diaz-Gil C, et al. (2015). Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae* (*Amaranthaceae*). Syst Appl Microbiol 38:406-416.

Moyes AB, Kueppers LM, Pett-Ridge J, et al. (2016). Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. New Phytol 210:657-668.

Nakagawa T, Kawaguchi M. (2006). Shoot-applied MeJA suppresses root nodulation in *Lotus japonicus*. Plant Cell Physiol 47:176-180.

Newman KL, Chatterjee S, Ho KA, Lindow SE. (2008). Virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-to-cell signaling factors. Mol Plant Microbe In 21:326-334.

Gottel NR, Castro HF, Kerley MK, et al. (2011). Distinct microbial communities within the endosphere and rhizosphere of Populus deltoides roots across contrasting soil types. Appl Environ Microb 77:5934-5944.

Olivares F, James E, Baldani JI, Döbereiner J. (1997). Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirilium*. New Phytol 135:723-737.

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC. (2009). Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308-316.

Pieterse CM, Zamioudis C, Berendsen RL, et al. (2014). Induced systemic resistance by beneficial microbes. Annu Rev Phytopathol 52: 347-375.

Piromyou P, Songwattana P, Greetatorn T, et al. (2015). The Type III Secretion System (T3SS) is a determinant for rice-endophyte colonization by non-photosynthetic *Bradyrhizobium*. Microbes Environ 30:291.

Prasertsincharoen N, Constantinoiu C, Gardiner C, et al (2015). Effects of colonization of the roots of domestic rice (*Oryza sativa* L. cv. Amaroo) by *Burkholderia pseudomallei*. Appl Environ Microb 81:4368-4375.

Puri A, Padda KP, Chanway CP. (2016). Seedling growth promotion and nitrogen fixation by a bacterial endophyte *Paenibacillus polymyxa* P2b-2R and its GFP derivative in corn in a long-term trial. Symbiosis 69:123-129.

Qin S, Zhang YJ, Yuan B, et al. (2014). Isolation of ACC deaminase-producing habitat-adapted symbiotic bacteria associated with halophyte *Limonium sinense* (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. Plant Soil 374:753-766.

Reinhold B, Hurek T. (1989). Location of diazotrophs in the root interior with special attention to the kallar grass association. In: Nitrogen fixation with non-legumes. Springer. p. 209-218.

Reinhold-Hurek B, Bünger W, Burbano CS, et al. (2015). Roots shaping their microbiome: Global hotspots for microbial activity. Annu Rev Phytopathol 53:403-424.

Reinhold-Hurek B, Hurek T. (2011). Living inside plants: bacterial endophytes. Curr Opin Plant Biol 14:435-443.

Reinhold-Hurek B, Maes T, Gemmer S, et al. (2006). An endoglucanase is involved in infection of rice roots by the not-cellulose-metabolizing endophyte *Azoarcus* sp. strain BH72. Mol Plant Microbe In 19:181-188.

Ren G, Zhang H, Lin X, et al. (2015a). Response of leaf endophytic bacterial community to elevated CO_2 at different growth stages of rice plant. Front Microbiol 6.

Ren G, Zhu C, Alam MS, et al. (2015b). Response of soil, leaf endosphere and phyllosphere bacterial communities to elevated CO_2 and soil temperature in a rice paddy. Plant Soil 392:27-44.

Rodriguez-Blanco A, Sicardi M, Frioni L. (2015). Plant genotype and nitrogen fertilization effects on abundance and diversity of diazotrophic bacteria associated with maize (*Zea mays* L.). Biol Fert Soils 51:391-402.

Rolli E, Marasco R, Vigani G, et al. (2015). Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environ Microbiol 17:316-331.

Rosenblueth M, Martínez-Romero E. (2006). Bacterial endophytes and their interactions with hosts. Mol Plant Microbe In 19:827-837.

Rungin S, Indananda C, Suttiviriya P, et al. (2012). Plant growth enhancing effects by a siderophore-producing endophytic streptomycete isolated from a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105). A Van Leeuw J Microb 102: 463-472.

Sessitsch A, Hardoim P, Döring J, et al. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol Plant Microbe In 25:28-36.

Shahzad R, Waqas M, Khan AL, et al. (2016). Seed-borne endophytic *Bacillus amyloliquefaciens* RWL-1 produces gibberellins and regulates endogenous phytohormones of *Oryza sativa*. Plant Physiol Bioch 106:236-243.

Sheibani-Tezerji R, Rattei T, Sessitsch A, et al. (2015). Transcriptome profiling of the endophyte *Burkholderia phytofirmans* PsJN indicates sensing of the plant environment and drought stress. MBio.6:e00621-00615.

Shen SY, Fulthorpe R. (2015). Seasonal variation of bacterial endophytes in urban trees. Front Microbiol 6.

Straub D, Rothballer M, Hartmann A, Ludewig U. (2013). The genome of the endophytic bacterium *H. frisingense* GSF30(T) identifies diverse strategies in the *Herbaspirillum* genus to interact with plants. Front Microbiol 4.

Su F, Jacquard C, Villaume S, et al. (2015). *Burkholderia phytofirmans* PsJN reduces impact of freezing temperatures on photosynthesis in *Arabidopsis thaliana*. Front Plant Sci 6.

Subramanian P, Mageswari A, Kim K, et al. (2015). Psychrotolerant endophytic *Pseudomonas* sp. strains OB155 and OS261 induced chilling resistance in tomato plants (*Solanum lycopersicum* Mill.) by activation of their antioxidant capacity. Mol Plant Microbe In 28:1073-1081.

Taghavi S, Van Der Lelie D, Hoffman A, et al. (2010). Genome sequence of the plant growth promoting endophytic bacterium *Enterobacter* sp. 638. PLoS Genet 6:e1000943.

Tian BY, Cao Y, Zhang KQ. (2015). Metagenomic insights into communities, functions of endophytes, and their associates with infection by root-knot nematode, *Meloidogyne incognita*, in tomato roots. Sci Rep 5:17087.

Toumatia O, Compant S, Yekkour A, et al. (2016). Biocontrol and plant growth promoting properties of *Streptomyces mutabilis* strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization. S Afr J Bot 105:234-239.

Trdá L, Fernandez O, Boutrot F, et al. (2014). The grapevine flagellin receptor VvFLS2 differentially recognizes flagellin-derived epitopes from the endophytic growth-promoting bacterium *Burkholderia phytofirmans* and plant pathogenic bacteria. New Phytol 201:1371-1384.

Truyens S, Weyens N, Cuypers A, Vangronsveld J. (2015). Bacterial seed endophytes: genera, vertical transmission and interaction with plants. Environ Microbiol Rep 7:40-50.

Tseng TT, Tyler BM, Setubal JC. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiol 9 (Suppl 1):S2.

Tsurumaru H, Okubo T, Okazaki K, et al. (2015). Metagenomic analysis of the bacterial community associated with the taproot of sugar beet. Microbes Environ 30:63-69.

Tudela D, Primo-Millo E. (1992). 1-Aminocyclopropane-1-carboxylic acid transported from roots to shoots promotes leaf abscission in Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) seedlings rehydrated after water stress. Plant Physiol 100:131-137.

Upreti R, Thomas P. (2015). Root-associated bacterial endophytes from *Ralstonia solanacearum* resistant and susceptible tomato cultivars and their pathogen antagonistic effects. Front Microbiol 6.

Verma V, Singh S, Prakash S. (2011). Bio-control and plant growth promotion potential of siderophore producing endophytic Streptomyces from *Azadirachta indica* A. Juss. J Basic Microbiol 51:550-556.

Vorholt JA. (2012). Microbial life in the phyllosphere. Nat Rev Microbiol 10:828-840.

Wright KM, Chapman S, McGeachy K, et al. (2013). The endophytic lifestyle of *Escherichia coli* O157: H7: quantification and internal localization in roots. Phytopathology103:333-340.

Yu X, Yang J, Wang E, et al. (2015). Effects of growth stage and fulvic acid on the diversity and dynamics of endophytic bacterial community in *Stevia rebaudiana* Bertoni leaves. Front Microbiol 6.

Zachow C, Jahanshah G, de Bruijn I, et al. (2015). The novel lipopeptide poaeamide of the endophyte *Pseudomonas poae* RE* 1-1-14 is involved in pathogen suppression and root colonization. Mol Plant Microbe In 28:800-810.

Zeiller M, Rothballer M, Iwobi AN, et al. (2015). Systemic colonization of clover (*Trifolium repens*) by *Clostridium botulinum* strain 2301. Front Microbiol 6.

Zhang Y, He L, Chen Z, et al. (2011). Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus*. Chemosphere 83: 57-62.

Zhao Q, Shen Q, Ran W, et al. (2011). Inoculation of soil by *Bacillus subtilis* Y-IVI improves plant growth and colonization of the rhizosphere and interior tissues of muskmelon (*Cucumis melo* L.). Biol Fert Soils 47:507-514.

Zhou JY, Yuan J, Li X, et al. (2015). Endophytic bacterium-triggered reactive oxygen species directly increase oxygenous sesquiterpenoid content and diversity in *Atractylodes lancea*. Appl Environ Microb 82:1577-1585.

Zúñiga A, Poupin MJ, Donoso R, et al. (2013). Quorum sensing and indole-3-acetic acid degradation play a role in colonization and plant growth promotion of *Arabidopsis thaliana* by *Burkholderia phytofirmans* PsJN. Mol Plant Microbe Interact 26:546-553.

Appendix 2 Book Chapter: "Emerging culture-independent tools to enhance our understanding for soil microbial ecology"

Hongwei Liu, Hooman Mirzaee, Vivian Rincon-Florez, Richard Moyle, Marie Spohn, Lilia C Carvalhais, Peer M Schenk

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1 Introduction

Soil bacteria, fungi, protists and archaea constitute the most diverse living communities on Earth and provide essential ecosystem services to life on this planet. However, the genetic information that they encode remained largely unexplored until recently. The main reason for this is that many soil microorganisms cannot be cultured using standard techniques [Ritz, 2007; Clardy *et al.,* 2006] and methods that enable culture-independent exploration of their genomes have only recently become available [Jones *et al.,* 2009; Scholz *et al.,* 2011; Lauber *et al.,* 2009]. While these new approaches gain a lot of attention, it appears that culture-independent techniques can also be very well complemented with classical microbiological culture-dependent techniques, and in some cases impressive numbers of soil microbes have been isolated from cultures. For example, a novel high-throughput *in situ* cultivation platform has been developed to cultivate and isolate hitherto uncultivated microbial species from a variety of environments, including soil. In an 'isolation chip' (ichip), several hundred miniature diffusion chambers can be colonised in a single environmental cell, enabling the investigation of a large and diverse array of previously inaccessible microorganisms [Kaeberlein *et al.*, 2002; Nichols *et al.*, 2010]. In this new method significantly more species were grown in comparison with standard media. As a one step process, 'unculturable' microorganisms inside the ichip generated pure colonies under the conditions that they had been collected from the environment [Nichols *et al.*, 2010]. Furthermore, a recent study that simply used a variety of different cultivation media shows that up to 70% of soil microbes associated with *Arabidopsis* plant roots can be cultured and match the data from culture-independent next generation 16 ribosomal RNA (rRNA) gene amplicon sequencing [Bai *et al.,* 2015]. This chapter provides cases of emerging culture-independent techniques that will enhance our understanding for soil microbial ecology. Soil ecosystem function, biodiscovery from soil microbes and plant-microbe interactions are just some of the areas that will benefit from these new approaches.

It can be expected that many important functions and compounds of soil microbiomes will be discovered in the near future. Cultured microbial strains have already been the source of numerous natural products, but the cryptic uncultivated majority is believed to produce an immense and vastly unexplored source of bioactive molecules [Hibbing *et al.,* 2010], in particular by members of the actinomycetes [Watve *et al.,* 2001]. Similarly, peptide bacteriocins (Class I and II) are abundant in bacterial microbial soil ecosystems, including the soil surrounding plant roots (rhizosphere), and genome searches reveal the presence of potential bacteriocin genes in most bacteria [Montesinos, 2007; Dirix *et al.,* 2004; Nes and Johnsborg, 2004; Holtsmark *et al.,* 2008].

Soil microbiomes have been influenced by humans in many ways since the advent of agriculture. These include (1) soil amendments (additives to soil, such as fertilisers, pesticides, charcoal, manure or other organic matter sources), (2) soil cultivation (e.g. tillage) and (3) crop rotation or mixed crop systems (e.g. legumes biofertilise the soil or the use of allelopathic plants). While soil-borne pathogens, such as *Fusarium*, *Pythium* and *Phytophthora* ssp. often receive a lot of attention, the vast majority of soil microbes can be considered neutral (commensals) or beneficial to plants. Our experiments have shown that plants grown in axenic soil may only produce half the biomass than in the presence of soil microbes [Carvalhais *et al.,* 2013a]. Beneficial soil microorganisms play a major role in plants for nutrient acquisition (e.g. by N fixation or P solubilisation) and disease suppression (e.g. by production of siderophores, antimicrobial compounds or anti-fungal chitinases). For this reason, plants release large amounts of organic carbon (sugars and organic acids) into the rhizosphere to recruit soil microbes that provide benefits to the plants. The direct addition of certain or mixed microorganisms (e.g. *Bacillus subtilis* or *Trichoderma*) to soil has also been practised with varying results to improve plant nutrition and/or disease resistance [Cao *et al.,* 2011; Kavoo-Mwangi *et al.,* 2013]. There is mounting evidence that plants can selectively attract and maintain rhizosphere microbes by root exudates to gain benefits, but the chemical language and services from these types of soil microbiome manipulations are often still poorly understood or unknown, especially for commercial crop plants. The well-studied legume-rhizobia interactions provide a good example for a chemical language where specific compounds attract specific rhizobacteria [Fierer *et al.,* 2007; Cooper, 2007]. It can be expected that similar common principles between compounds, attracted microbe and function can be established for other parts of the rhizosphere microbiome, although some of them will be less specific. Figure 1 provides an overview of beneficial and parasitic plant-microbe interactions in the rhizosphere and phyllosphere. The following paragraphs provide examples of emerging technologies that will enhance our understanding of soil microbiomes in this and other areas.

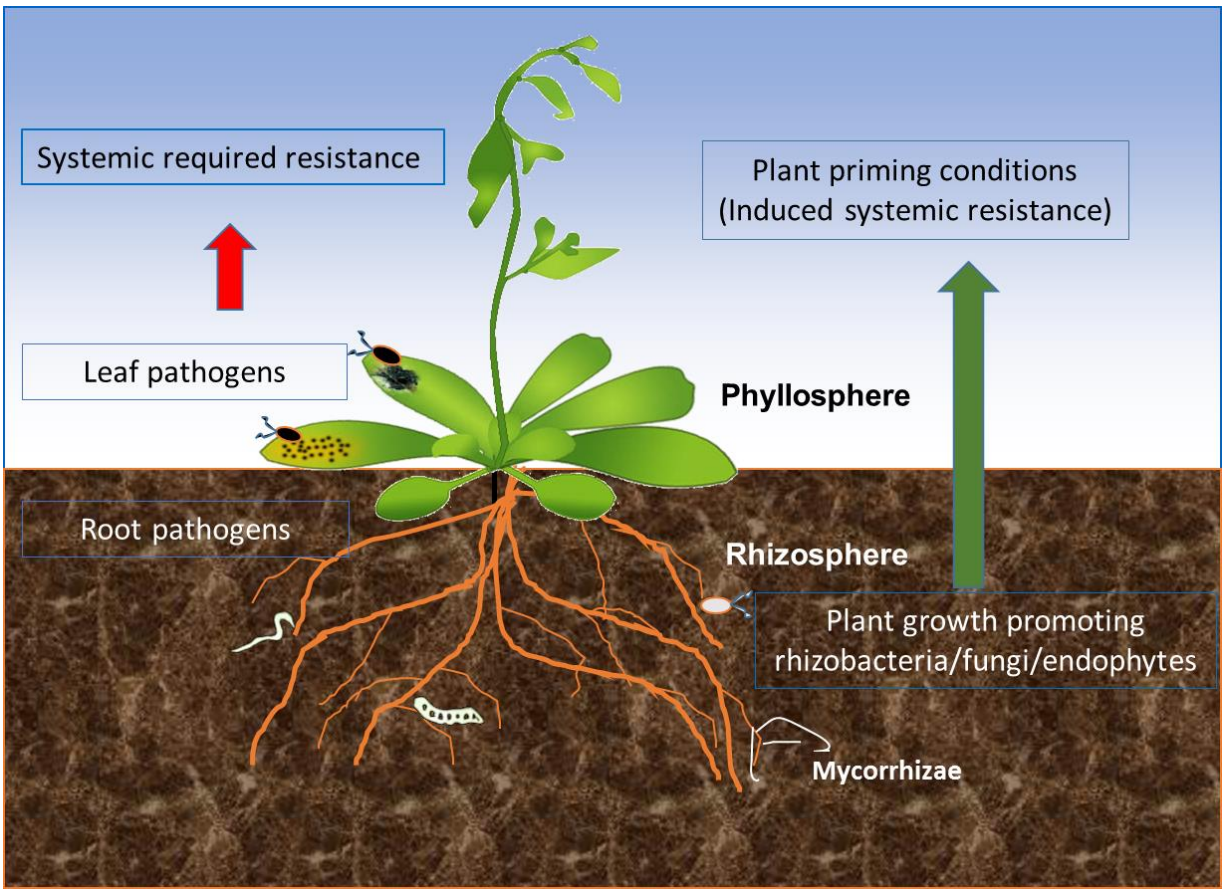

Fig. 1 Overview of plant-microbe interactions in the rhizosphere and phyllosphere. Plant growth-promoting microbes are attracted to the rhizosphere and phyllosphere by root and leaf exudates, respectively. In return they make nutrients available to plants, control pathogens, or influence plant development, disease resistance and stress resilience by manipulating plant hormone signalling.

2. Characterisation of soil microbial genes and enzymes

2.1 Quantitative PCR

Real-time quantitative PCR (Q-PCR) or reverse transcriptase Q-PCR (qRT-PCR) are culture-independent methods to quantify the abundance of genes in the environment [Smith and Osborn 2009]. Gene abundance measurements can be used for taxonomic and functional analysis of microbial communities colonising environmental samples [Fierer *et al*., 2005; Torsvik and Øvreås 2002). Q-PCR is a highly sensitive, accurate and quick method that allow the analysis of several samples with different genes in the same Q-PCR run [Heid *et al*., 1996]. However, this method can only be used for the quantification of known sequences and requires prior knowldege for primer design. Q-PCR/qRT-PCR is based on fluorescence chemistries using either intercalating fluorescent probes of TaqMan or SYBR Green to detect the accumulation of amplicons during PCR cycles [VanGuilder *et al*., 2008]. TaqMan chemistry requires specific hybridisation between probe and

target to produce a fluorescent signal. Therefore, Q-PCR using TaqMan chemistry has higher specificity than using SYBR green. TaqMan also allows parallel amplifications of two different sequences in one reaction by using distinguishable reporter dyes of probes. In contrast, the SYBR Green dye binds to any double-stranded DNA and does not require a probe to produce a fluorescence signal, and this reduces running costs for assays [VanGuilder *et al*., 2008]. One drawback of using SYBR Green dye is that false positive signals may be generated during PCR due to nonspecific amplifications or DNA contamination [Smith and Osborn 2009]. To achieve accurate quantification in a Q-PCR assay, optimisation for template concentration and the annealing temperature of gene-specific primers prior to Q-PCR is necessary.

Q-PCR that targets the 16S rRNA genes of genomic DNA has been proposed to evaluate relative abundances of some taxonomic bacterial groups in microbial ecology [De Gregoris *et al.*, 2011; Fierer *et al*., 2005]. Using this method, Liu *et al.* [2016a,b] evaluated the impacts of one-time strategic tillage on the abundance of five bacterial taxa (Actinobacteria, α and γ Proteobacteria, Firmicutes and Bacteriodetes) of microbial communities in Australian long-term no-till soils. Another important application of Q-PCR/qRT-PCR is the profiling of functional genes (e.g. associated with nitrification and denitrification) in environmental samples. The understanding of nitrogen (N) and carbon (C) cycles is an important theme for microbial ecological research [Paul 2014]. For instance using Q-PCR, Harter *et al.* [2014] quantified the abundance and expression (using reverse transcription) of soil microbial nitrogen fixation (*nifH*), nitrification (*amoA*) and denitrification (*nirK*, *nirS* and *nosZ*) after biochar addition in soil, and they found N_2O emissions was reduced and N_2-fixing microorganisms were increased in abundance. This finding contributes to a better understanding of the impact of biochar on the N cycling of soil microbial communities. Unfortunately, qRT-PCR can be challenging as it requires enough good quality mRNA from environmental samples, e.g. soil samples. This often involves the use of special protocols to remove phenolics, carbohydrates and humic acids from soil, all of which can inhibit reverse transcriptases and/or polymerases.

2.2 Soil zymography

An interesting emerging imaging technique for localising and quantifying enzyme activities in soil is zymography [Spohn *et al.,* 2013; Spohn and Kuzyakov, 2013]. The advantages of soil zymography are that it is an *in situ* method, and therefore no sample preparation such as soil sieving or enzyme extraction is necessary. The method is non-destructive, which means that the same soil

can be measured several times [Spohn and Kuzyakov, 2014; Hofmann et al., 2016], which is very useful for the determination of changes in enzyme activity over time. Moreover, it allows to determine the distribution of enzyme activity at a small scale since it has a resolution of about 2 mm. Soil zymography is based on the incubation of the soil attached to an agarose gel that is covered by a nylon membrane coated with a substrate that becomes fluorescent once it gets hydrolyzed. After the incubation, which typically takes between 20 and 40 minutes, the fluorescence is made visible by excitation with UV light, and a photograph of the fluorescent membrane is taken. Calibration is performed with nylon membranes coated with solutions of standards [Spohn and Kuyzakov, 2014]. Soil zymography works for a large range of hydrolases [Spohn et al., 2013; Spohn and Kuzyakov, 2014]. The method has been especially useful for determining the distribution of enzyme activity in the rhizosphere [Spohn and Kuzyakov, 2013, 2014], and it has already been combined with other imaging methods, such as ^{14}C imaging [Spohn and Kuzyakov 2013] and fluorescent *in situ* hybridisation [Spohn *et al.,* 2015] to gain insights into rhizosphere processes (Fig. 2).

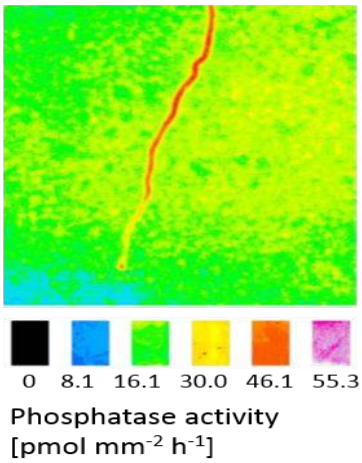

Fig. 2 Example of using soil zymography to map and quantify phosphatase activity in the rhizosphere along a wheat root (*Triticum aestivum*) without P fertiliser addition to the soil. The comparison to calibration gels shown below the false colour image, enables quantification of enzymatic activity.

3 Microbial community profiling

3.1 Fluorescence in situ hybridisation

Fluorescence *in situ* hybridisation (FISH) as a cytogenetic technique has been used for more than 30 years [Levsky and Singer 2003; Wilkinson 1998]. It has become one of the most useful techniques in microbial ecology to visualise archaeal and bacterial cells in biospheres and does not

require prior cultivation [Amann *et al*., 2001]. The traditional FISH technique relied on 16S rRNA as a probe target for phylogenetic identification of microbes as 16S rRNA exists in all prokaryotic cells with high copy numbers. Typical FISH entails the procedures of cell fixation and permeabilisation, hybridisation, washing steps, and the detection of fluorescence signals [Pernthaler *et al*., 2002]. Microbial cells are fixed and their DNA and RNA are preserved. Those microbial cells with permeabilised conditions allow the nucleic acid probes access and hybridisation to the target site. The detection of cells of interest in environmental samples is often achieved using epifluorescence or laser scanning microscopy or flow cytometry for spatial distribution, quantification and further studies. FISH has several advantages over other fingerprinting techniques, such as terminal reaction fragment length polymorphism (T-RFLP), 16S rRNA sequencing or phospholipid-linked fatty acid methyl esters (PLFA): (1) FISH allows the detection of both culturable and non-culturable microorganisms; (2) it enables phylogenetic identification of individual cells *in situ* in their natural environment and may assist to discover their functions in ecosystems. FISH is well-established and widely used in microbial ecology to identify microorganisms [Moter and Göbel 2000]. As a very useful tool for environmental microbiological studies, FISH has spawned numerous applications. Over the past decade, more and more FISH techniques have been applied to complex environmental samples (e.g. marine environments, plant and soil environments, biosphere [Amann *et al*., 2001]). For instance, biofilms contain a diverse range of bacterial species. Performing FISH allows the visualisation of specific bacterial taxa in biofilms by using bacteria-specific FISH DNA probes. CARD-FISH has been recently used to confirm the presence of Actinobacteria on whole root segments of *Arabidopsis* plants. It was found that on the root surface of *Arabidopsis*, Actinobacteria were visually detected in higher numbers while the abundance of Bradyrhizobiaceae was lower using the probe HGC69a [Lundberg *et al*., 2012]. Remus-Emsermann *et al.* [2014] established a FISH protocol for the measurement of spatial distribution of different main phylogenetic lineages of bacterial communities on the leaf surface of *Arabidopsis*. They found that most of the bacterial biomass was composed of taxa affiliated to Betaproteobacteria, Alphaproteobacteria and Actinobacteria. About one-third of the bacteria found in the phylloplane exhibited infrared autofluorescence, which could be from aerobic anoxygenic phototrophs [Remus-Emsermann *et al*., 2014].

3.2 Phylogenetic marker gene sequencing

High-throughput deep sequencing, or next generation sequencing is being widely used for the evaluation of microbial diversity in the environment. Compared to other culture-independent

methods, e.g. T-RFLP, phylogenetic gene sequencing allows the exploration of biodiversity and abundance of environmental samples with reliable taxonomic units up to the genus level. It presents a descriptive picture for microbial ecology studies and abundance fluctuations of microbes with known functions enable functional analyses of microbial communities [Bartram *et al.,* 2011; Carvalhais *et al.,* 2012; Simon and Daniel, 2011].

16S rRNA deep sequencing has been extensively used to detect the composition and diversity of archaeal and bacterial genera in soil samples. For instance, 16S rRNA gene amplicon pyrosequencing has been used for bacterial community profiling in different compartments of *Arabidopsis* including rhizosphere soil and in roots (endophytic) [Lundberg *et al.,* 2012; Bao *et al.,* 2015]. Endophytic bacteria displayed an overrepresentation of Actinobacteria and Proteobacteria for two different soil types tested. Our previous studies also found that elevated level of jasmonate signalling pathway in *Arabidopsis* altered the composition but not the α diversity in the associated rhizosphere bacterial communities [Carvalhais *et al.,* 2013b]. More recently, 18S rRNA and ITS amplicon deep sequencing have been used for the profiling of soil eukaryotic microbial communities to cover protists and fungi [Bates *et al.,* 2013; Hugerth *et al.,* 2014]. However, the nuclear ribosomal internal transcribed spacer (ITS) region of eukaryotes is considered to be more suitable for phytogenetic profiling of eukaryotes as it shows a higher variability [Schoch *et al.,* 2012; Adl *et al.,* 2014]. ITS primers are now more commonly used for amplicon sequencing to reveal fungal and protistic diversity in soils and this area of research is expected to grow.

4 High-throughput (next-gen) sequencing technologies

First generation sequencing technology, better known as Sanger sequencing, was used to complete the first bacterial genome sequence in 1995 [Fleischmann *et al.,* 1995]. 454 Life Sciences launched a sequencing-by-synthesis second generation sequencing platform in 2005. This advance in high-throughput, massively parallel sequence analysis permitted sequencing of bacterial genomes in a matter of days.

Today, entire microbiomes are analysed using next generation sequencing technologies which possess a combination of longer read lengths, higher throughput per run and higher depth of coverage. A number of next generation sequencing platforms exist, each with their own strengths and weaknesses. The 454 GS FLX+ platform is particularly useful at generating long reads in under a day (up to 1000 bp in length), but has relatively low throughput of ~700 Mb per run, can have

relatively high error rates (particularly at homopolymeric tracts of sequence), and is relatively expensive compared to other platforms. The Illumina HiSeq2500 platform is higher throughput with up to 600 Gb per run and has a lower reagent cost, but takes over a week per run and generates shorter read lengths of up to 150 bp. The SOLiD platform is also high throughput, generating ~320 Gb per run with high accuracy, but generates shorter read lengths of up to 75 bp. Thus, the appropriate selection of next generation sequencing platform depends upon the particular aims of each experiment. In some cases combinations of platforms are chosen, for example pairing the 454 platform with the Illumina platform provides both long reads for sequence scaffold assembly and high depth of coverage respectively.

4.1 Metagenomics

Metagenomics involves investigating simultaneously multiple genomes present in the DNA of cohabiting microbial populations, recovered directly from the environment [Thomas *et al.*, 2012] Currently this is mainly achieved by direct random shotgun sequencing [Tyson *et al.*, 2004]. The decline in the cost of high-throughput sequencing technologies is the main reason why recently metagenome datasets have expanded massively. One of the applications of metagenome analyses is to assess taxonomic information of the sample being investigated. Marker genes which are well known to provide phylogenetic information are usually targeted in the analysis. The other application is to understand the potential ecological processes ongoing in the sample. Compared to cultivation-based methods, metagenomics approaches are more likely to reveal unbiased insights into microbial community composition and function given that many of the microbes cannot be cultivated in standard laboratory culture medium [Hugenholtz and Tyson, 2008]. In a single environmental sample, organisms from distinct domains typically co-exist, including viruses, bacteria, archaea, and microbial eukaryotes. However, the intricate nature of the ecological information contained in most ecosystems favours the study of individual domains, most often combining bacteria and archaea. The main constraint involved in including eukaryotes in metagenomics analyses is that their genomes contain a significant portion of non-coding genetic material. Another limitation stems from the fact that eukaryotic genomes are much more complex because they usually have two (or more) sets of chromosomes [Kunin *et al.*, 2008]. Metagenomics provides information on the phylogenetic types, gene functions, and interactions between different organisms co-inhabiting the environmental sample under evaluation. Comparisons between metagenomes have shed light into adaptive microbial strategies to thrive in environments with different chemical and physical properties and into the abundance of distinct gene families in such

environments [Tringe *et al.*, 2005; Dinsdale *et al.*, 2008; Delmont *et al.*, 2011]. To properly interpret the data obtained in metagenomics studies, detailed data on the chemical, biological and physical attributes need to be put into context, as well as suitable experimental design and statistics [Knight *et al.*, 2012].

Sequencing of environmental DNA for metagenomics analyses can be performed either deeply or shallowly, depending on the purpose of the investigation. Deep sequencing is especially required to detect rare taxa. Rare microbes can be essential for the functioning of soil ecosystems, given that these organisms are pivotal for processes like nitrogen fixation and methanogenesis [Falkowski *et al.*, 2008; Thauer *et al.*, 2008]. However, microbial groups that are rare in one environment may be abundant in another. For example, nitrogen fixing organisms are abundant in the root nodule of a leguminous plant and may be rare in a soil sample. Shallow sequencing indicates the most abundant microbial groups present at the time of the sampling. Given that the most dominant microbial populations are possibly the most functionally relevant under standard conditions, in studies which aim at assessing the most representative organisms and processes, shallow sequencing should suffice [Knight *et al.*, 2012]. Nevertheless, information on the dynamics of microbial communities can only be gained if multiple samples are taken on a time course and across a spatial gradient [Knight *et al.*, 2012].

A series of bioinformatics methods is then used to treat and analyse the data after the sequencing step has been concluded. In the case of environments with highly complex microbial communities, such as most soil samples, often the sequencing depth is not enough to perform a metagenome assembly, and therefore a gene-centric approach is used for the analysis. Bioinformatics methods include quality control, clustering (for gene-centric approaches) or assembly (in case a reference genome is available), binning and taxonomic classification, functional annotation and metabolic reconstruction. A wide range of high quality reviews on metagenomics analysis tools is available [for more detailed information please refer to Kim *et al.*, 2013; Bragg and Tyson, 2014; Lindgreen *et al.*, 2016].

4.2 Metatranscriptomics

Metatranscriptomic approaches (also referred to as RNASeq of microbiomes) reveal a functional snapshot of the ongoing ecological processes and active taxa in the environmental sample at the transcriptional level. Apart from removing reads derived from rRNA,

metatranscriptomic approaches involve exactly the same analytical steps to metagenomic approaches after high-throughput sequencing. However the starting material extracted from environmental samples is RNA instead of DNA. As a consequence, when obtaining information concerning the taxonomic affiliation of members of the microbial communities, the results reveal a snapshot of the active members of the community given RNA molecules are a product of gene transcription and these molecules have short half-lives compared to DNA. Half-lives of RNA samples vary from minutes to seconds (Deutscher, 2006]. During sampling, it is essential to prevent RNA from being degraded. Procedures that are recommended for this purpose include snap-freezing samples at -80ºC immediately after collection or using a commercial RNA preservation solution, such as the LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, Carlsbad, CA). A common constraint of metatranscriptomic approaches is the isolation of sufficient high quality RNA for the downstream analysis. Challenges arise mainly from RNA degradation by RNases, poor cell lysis and, in the case of soil samples, adsorption of RNA to soil particles. High salt concentration in extraction buffers are often used to inactivate RNases. Lowering the pH of extraction buffers prevents adsorption to soil particles [Chomczynski and Sacchi, 1987]. Numerous extraction kits for RNA isolation of environmental samples are available commercially. These include E.Z.N.A.® Soil RNA kit (Omega Bio-tek, Norcross, GA, USA), PowerSoil™ Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA), FastRNA® Pro Soil-Indirect kit (MP Biomedicals, Solon, OH, USA), FastRNA® Pro Soil-Direct kit, ZR Soil/FEcal RNA MicroPrep™, ZR Plant RNA MiniPrep™ and Direct-zol™ RNA kits (Zymo Research, Orange, CA, USA).

Another common attribute of soil-derived RNA is the presence of compounds that have similar chemical properties to nucleic acids and inhibit enzymes that act in subsequent steps to the RNA isolation, including reverse transcriptases. Examples of such compounds include humic and fulvic acids. Commercial kits are available for cleaning-up, removing inhibitors and concentrating RNA samples: RNA Clean & Concentrator™, OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Orange, CA, USA) and PureLink® RNA Mini Kit (Ambion Inc., Austin, TX, USA). Other methods for removal of inhibitors are reviewed in Carvalhais *et al.*, [2012].

Methods for isolating RNA from environmental samples usually extract total RNA, which is mostly composed of ribosomal RNA (95-99% of the total RNA, [He *et al.*, 2010]. For functional studies it is paramount to enrich for messenger RNA (mRNA). To this purpose, various methods have been developed: i) duplex specific nuclease (DSN, [Yi *et al.*, 2011]; ii) exonuclease treatment (mRNA-ONLY Prokaryotic mRNA Isolation kit, EPICENTRE Biotechnologies, Madison; USA); iii) subtractive hybridisation (MICROBExpress Bacterial mRNA Enrichment Kit, Ambion, USA; Ribo-Zero rRNA Removal Kit (Bacteria), Illumina [Stewart *et al.*, 2010]; iv) size separation by gel

electrophoresis [McGrath *et al.*, 2008]. Messenger RNA amplification may be required as obtaining sufficient amounts of this molecule for downstream applications is challenging. An example of an efficient commercial kit available for mRNA amplification is the MessageAmp II-Bacteria aRNA amplification kit (Ambion, USA). This kit includes a polyadenylation step using a *E. coli* poly(A) polymerase. The poly(A)-tailed RNA is then reverse-transcribed using an oligo(dT) primer containing a T7 promoter. Other commercial kits are also available, such as SMART®mRNA Amplification kit (Clontech, Palo Alto, CA, USA), Arcturus™ RiboAmp® PLUS Kit (Applied Biosystems, Foster City, CA, USA) and SeqPlex RNA Amplification Kit (Sigma-Aldrich). Even if mRNA enrichment methods are used, a substantial portion of the reads derive from rRNA after high throughput sequencing [51-60%; Stewart *et al.*, 2010]. Several bioinformatics tools are available for identifying and removing of rRNA-derived sequences. These include riboPicker [Schmieder *et al.*, 2012], SortMeRNA [Kopylova *et al.*, 2012] and Infernal [Nawrocki and Eddy, 2013].

5 Biodiscovery of compounds from soil microbiomes

Soil microorganisms are untapped resources for new biotechnological compounds which could be used in many industries. For example, plant growth promoting rhizobacteria and other beneficial microbes are able to control plant diseases through mechanisms like competition, antagonism through the production of antibiotics, antimicrobial peptides and siderophores; induction of disease resistance and improvement of nutrient uptake (Fig. 1; Anith *et al.*, 2004; Babalola, 2010; Maksimov *et al.*, 2011]. While there is a high abundance microorganisms in soil samples, most are not culturable in standard laboratory culture media [Schmeisser *et al.*, 2007; Walsh and Duffy, 2013] and may only produce certain compounds in the presence of other microbes and their products. For this reason, it is possible that a significant amount of new bioactive compounds from unculturable microbes remain unknown. Recently, using a novel approach in culturing and screening of new antibiotics called 'iChip' (see above), 25 antibiotics have been discovered. They have been shown to be effective against multiple drug resistant *Staphylococcus aureus* or *Mycobacterium tuberculosis* [Ling *et al.*, 2015; Nichols *et al.*, 2010].

5.1 Bacteriocin-targeted PCR using degenerate primers

Proteins, enzymes, and short peptides are encoded in the DNA. AMPs, which can have inhibitory effects against human and plant pathogens, are synthesised in a wide range of organisms from all kingdoms [Keymanesh *et al.*, 2009; Maróti *et al.*, 2011; Nakatsuji and Gallo, 2012]. To

find new AMPs synthesised by unculturable microbes, conserved regions in the coding DNA sequences can be used in PCR-based approaches using conserved degenerate primers. A phylogram can be generated using AMP-coding sequences from publically available databases by bioinformatics softwares and degenerate primers can be designed targeting conserved regions from aligned sequences from the different clusters of the phylogram. An example of a template derived from an environmental sample for PCR using degenerate primers is the DNA isolated from soil samples. PCR products can then be ligated to suitable vectors for sequencing, protein expression and/or further functional characterisation.

5.2 Cloning and screening of expression libraries

Targeting the soil microbial transcriptome for biodiscovery of bioactive proteins or peptides is recommended when treatments or environmental conditions favors the detection of coding sequences of molecules of interest via the induction of transcription. However, several challenges associated with this approach need to be taken into account. For instance, as previously discussed, RNA has a short half-life. Since transcription and translation of archaea and bacteria occur simultaneously, mRNA degradation starts quickly after translation [Deutscher, 2006]; therefore, the chances of obtaining complete sequences of a target gene are reasonably low. To perform experiments targeting the environmental microbial transcriptomes, firstly total RNA needs to be extracted from soil or any other environmental samples, followed by cDNA synthesis. The latter includes a range of reverse degenerated primers which would target the gene of interest, for example AMPs such as bacteriocins. Similar as required for metatranscriptomics (see above), rRNA should be subtracted before cDNA synthesis. Finally, a suitable vector is essential to express the genes encoded in the cDNA in all reading frames and which should include an inducible promoter (e.g. IPTG-inducible T7 polymerase), start and stop codons. We have recently developed a new vector on the basis of RNA polymerase slippage [Baranov *et al.*, 2005; Penno *et al.*, 2006; Wagner *et al.*, 1990]. It contains a slippery region consisting of 12 thymine nucleotides and multiple stopping codons which allow directional cloning and simultaneous expression of cDNA in all reading frames.

Functional assays will be required to screen the clone libraries. For example to screen for AMPs, after obtaining colonies on selective medium, single colonies can be transferred to IPTG-containing medium for inhibition tests. On this medium, AMP genes, even with incomplete sequences cloned in the vector may be expressed and the growth of the colonies could be visibly

inhibited by the presence of large quantities of AMP in the cells. Colonies present in IPTG-free medium but absent in IPTG-containing medium can then be sequenced and new possible antimicrobial genes and related compounds can be found. Further pathogen growth inhibition assays can then be performed so that functional AMPs are identified.

6 Conclusion

Soil microbiomes are arguably the most complex ecosystems on Earth. A combination of culture-dependent and independent techniques can be used to elucidate the function of soil microbiomes. Many of the emerging DNA-, RNA-, protein- and metabolite-based tools have the potential to reveal a comprehensive picture of microbial community composition and its activity. These techniques, when coupled with high-throughput screening assays provide excellent platforms for biodiscovery approaches. It can be expected that, as more and more knowledge on soil microbiomes is accumulated, well developed bioinformatics tools will provide the path for future soil microbial ecology studies. However, many challenges remain in data mining and the elucidation of microbial functions. While correlations can be established relatively easily, to determine actual causality relationships requires well-defined experimental designs where specific questions can be tested experimentally.

References

Adl, S. M., Habura, A. and Eglit, Y., (2014). Amplification primers of SSU rDNA for soil protists. *Soil Biol. Biochem.,* 69, pp. 328-342.

Amann, R., Fuchs, B. M. and Behrens, S. (2001). The identification of microorganisms by fluorescence in situ hybridisation. *Curr. Opin. Biotechnol.,* 12, pp. 231-236.

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M. and Jones, J. B. (2004), Efficacy of plant growth-promoting rhizobacteria, acibenzolar-S-methyl, and soil amendment for integrated management of bacterial wilt on tomato, *Plant Dis.*, 88(6), pp. 669-673.

Babalola, O. (2010), Beneficial bacteria of agricultural importance, *Biotechnol Lett.*, 32(11), pp. 1559-1570.

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P. C., Spaepen, S., Remus-Emsermann, M., Hüttel, B., McHardy, A.C., Vorholt, J.A. and Schulze-Lefert P. (2015). Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature*. 528, pp. 364-369.

Baranov, P., Hammer, A., Zhou, J., Gesteland, R. and Atkins, J. (2005), Transcriptional slippage in bacteria: distribution in sequenced genomes and utilization in IS element gene expression, *Genome Biol.,* 6(3), pp. R25.

Bartram, A. K., Lynch, M. D. J., Stearns, J. C., Moreno-Hagelsieb, G. and Neufeld, J. D. (2011). Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Appl. Environ. Microbiol.*, 77, pp. 3846–3852.

Bates, S. T., Clemente, J. C., Flores, G. E., Walters, W. A., Parfrey, L. W., Knight, R. and Fierer, N. (2013). Global biogeography of highly diverse protistan communities in soil. *ISME J.,* 7(3), pp. 652-659.

Bragg, L. and Tyson, G. W. (2014). Metagenomics using next-generation sequencing. *Meth. Mol. Biol.,* 1096, pp. 183-201.

Cao, Y., Zhang, Z., Ling, N., Yuan, Y., Zheng, X., Shen, B. and Shen, Q. (2011). *Bacillus subtilis* SQR 9 can control Fusarium wilt in cucumber by colonizing plant roots. *Biol. Fert. Soils*, 47(5), pp. 495-506.

Carvalhais, L. C., Dennis, P. G., Badri, D. V., Tyson, G. W., Vivanco, J. M. and Schenk, P. M. (2013b). Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. PLoS One,8(2), p.e56457.

Carvalhais, L. C., Dennis, P. G., Tyson, G. W. and Schenk, P. M. (2012). Application of metatranscriptomics to soil environments. *J. Microbiol. Meth.,* 91, pp. 246-251.

Carvalhais, L. C., Muzzi, F., Tan, C. H., Hsien-Choo, J. and Schenk, P. M. (2013a). Plant growth in *Arabidopsis* is assisted by compost soil-derived microbial communities. *Front. Plant Sci.*, 4, pp. 235.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal. Biochem.,* 162, pp. 156-159.

Clardy, J., Fischbach, M. A. and Walsh, C. T. (2006). New antibiotics from bacterial natural products. *Nat. Biotechnol.*, 24(12), pp. 1541-1550.

Cooper, J. E. (2007). Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J. Appl. Microbiol.*, 103(5), pp. 1355-1365.

De Gregoris, T. B., Aldred, N., Clare, A. S. and Burgess, J. G. (2011) Improvement of phylum-and class-specific primers for real-time PCR quantification of bacterial taxa. *J. Microbiol. Meth.,* 86, pp. 351-356.

Delmont, T. O., Malandain, C., Prestat, E., Larose, C., Monier, J. M., Simonet, P. and Vogel T. M. (2011). Metagenomic mining for microbiologists. *ISME J.*, 5, pp. 1837-1843.

Deutscher, M. P. (2006), Degradation of RNA in bacteria: comparison of mRNA and stable RNA, *Nucl. Acids Res.*, 34(2), pp. 659-666.

Dinsdale, E. A., Edwards, R. A., Hall, D., Angly, F., Breitbart, M., Brulc, J. M., Furlan, M., Desnues, C., Haynes, M., Li, L., McDaniel, L., Moran, M. A., Nelson, K. E., Nilsson, C., Olson, R., Paul, J., Brito, B. R., Ruan, Y., Swan, B. K., Stevens, R., Valentine, D. L., Thurber, R. V., Wegley, L., White, B. A. and Rohwer, F. (2008). Functional metagenomic profiling of nine biomes. *Nature* 452, pp. 629-632.

Dirix, G., Monsieurs, P., Marchal, K., Vanderleyden, J. and Michiels, J. (2004). Screening genomes of Gram-positive bacteria for double-glycine-motif-containing peptides. *Microbiol.*, 150(5), pp.1121-1126.

Falkowski, P. G., Fenchel, T. and Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* 320, pp. 1034-1039.

Fierer, N., Jackson, J. A., Vilgalys, R. and Jackson, R. B. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.,* 71, pp. 4117-4120.

Fierer, N., Bradford, M. A. and Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology*, 88(6), pp. 1354-1364.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A. and Merrick, J. M. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science*, 269(5223), pp.496-512.

He, S. M., Wurtzel, O., Singh, K., Froula, J. L., Yilmaz, S., Tringe, S. G., Wang, Z., Chen, F., Lindquist, E. A., Sorek, R. and Hugenholtz, P. (2010). Validation of two ribosomal RNA removal methods for microbial metatranscriptomics. *Nat. Meth.* 7, pp. 807-858.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996). Real time quantitative PCR. *Genome Res.*, 6, pp. 986-994.

Hibbing, M. E., Fuqua, C., Parsek, M. R. and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.*, 8(1), pp. 15-25.

Hofmann, K. Y., Heuck, C. and Spohn, M. (2016) Phosphorus resorption by young beech trees and soil phosphatase activity as dependent on P availability. *Oecologia*, doi:10.1007/s00442-016-3581-x.

Holtsmark, I., Eijsink, V. G. and Brurberg, M. B. (2008). Bacteriocins from plant pathogenic bacteria. *FEMS Microbiol. Lett.*, 280(1), pp. 1-7.

Hugenholtz, P. and Tyson, G. W. (2008). Microbiology: metagenomics. *Nature* 455, pp. 481-483.

Hugerth, L. W., Muller, E. E., Hu, Y. O., Lebrun, L. A., Roume, H., Lundin, D., Wilmes, P. and Andersson, A. F. (2014). Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. *PLoS ONE,* 9(4), pp. e95567.

Kaeberlein, T., Lewis, K. and Epstein, S. S. (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment, *Science*, 296(5570), pp. 1127-1129.

Jones, R. T., Robeson, M. S., Lauber, C. L., Hamady, M., Knight, R. and Fierer, N. (2009). A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J.*, *3*(4), pp. 442-453.

Kavoo- Mwangi, A. M., Kahangi, E. M., Ateka, E., Onguso, J., Mukhongo, R. W., Mwangi, E. K. and Jefwa J. M. (2013). Growth effects of microorganisms based commercial products inoculated to tissue cultured banana cultivated in three different soils in Kenya. *Appl. Soil Ecol.* 64, pp. 152-162.

Keymanesh, K., Soltani, S. and Sardari, S. (2009). Application of antimicrobial peptides in agriculture and food industry, *World J. Microbiol. Biotechnol.*, 25(6), pp. 933-944.

Kim, M., Lee, K. H., Yoon, S. W., Kim, B. S., Chun, J. and Yi, H. (2013). Analytical tools and databases for metagenomics in the next-generation sequencing era. *Genomics Informatics* 11, pp. 102-113.

Knight, R., Jansson, J., Field, D., Fierer, N., Desai, N., Fuhrman, J.A., Hugenholtz, P., van der Lelie, D., Meyer, F., Stevens, R., Bailey, M.J., Gordon, J.I., Kowalchuk, G.A. and Gilbert, J.A. (2012). Unlocking the potential of metagenomics through replicated experimental design. *Nat. Biotechnol.* 30, pp. 513-520.

Kopylova, E., Noe, L. and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, pp. 3211-3217.

Kunin, V., Copeland, A., Lapidus, A., Mavromatis, K. and Hugenholtz, P. (2008). A bioinformatician's guide to metagenomics. *Microbiol. Mol. Biol. Rev.* 72, pp. 557-578.

Lauber, C. L., Hamady, M., Knight, R. and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.*, 75(15), pp. 5111-5120.

Levsky, J. M. and Singer, R. H. (2003). Fluorescence in situ hybridization: past, present and future. *J. Cell Sci.,* 116, 2833-2838.

Lindgreen, S., Adair, K. L. and Gardner, P. P. (2016). An evaluation of the accuracy and speed of metagenome analysis tools. *Sci. Rep.,* 6, pp. 19233.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schäberle, T. F., Hughes, D. E., Epstein, S. and Jones, M. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature*, 517(7535), pp. 455-459.

Liu, H., Carvalhais, L., Crawford, M., Dang, Y., Dennis, P. and Schenk, P. M. (2016a). Strategic tillage on a Grey Vertosol after fifteen years of no-till management had no short-term impact on soil properties and agronomic productivity. *Geoderma* 267, pp. 146-155.

Liu, H., Carvalhais, L. C., Rincon-Florez, V., Crawford, M., Dang, Y. P., Dennis, P. G., Schenk, P. M. (2016b). One-time strategic tillage does not cause major impacts on soil microbial properties in a no-till Calcisol. *Soil Tillage Res.,* 158, pp. 91-99.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Del Rio, T. G. and Edgar, R. C. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature*, 488(7409), pp. 86-90.

Maksimov, I. V., Abizgil'dina, R. R. and Pusenkova, L. I. (2011). Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens (review), *Appl. Biochem. Microbiol.*, 47(4), pp. 333-345.

Maróti, G., Kereszt, A., Kondorosi, É. and Mergaert, P. (2011). Natural roles of antimicrobial peptides in microbes, plants and animals, *Res. Microbiol.*, 162(4), pp. 363-374.

McGrath, K. C., Thomas-Hall, S. R., Cheng, C. T., Leo, L., Alexa, A., Schmidt, S. and Schenk, P. M. (2008). Isolation and analysis of mRNA from environmental microbial communities. *J. Microbiol. Meth.* 75, pp. 172-176.

Montesinos, E. (2007). Antimicrobial peptides and plant disease control. *FEMS Microbiol. Lett.*, 270(1), pp. 1-11.

Moter, A. and Göbel, U. B. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Meth.,* 41, pp. 85-112.

Nakatsuji, T., and Gallo R. L. (2012). Antimicrobial peptides: old molecules with new ideas. *J. Invest. Dermatol.*, 132(3), pp. 887-895.

Nawrocki, E. P. and Eddy, S. R. (2013). Infernal 1.1: 100-fold faster RNA homology searches. *Bioinformatics* 29, pp. 2933-2935.

Nes, I. F. and Johnsborg, O. (2004). Exploration of antimicrobial potential in LAB by genomics. *Curr. Opin. Biotechnol.*, 15(2), pp. 100-104.

Nichols, D., Cahoon, N., Trakhtenberg, E. M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K. and Epstein, S. S. (2010). Use of Ichip for high-throughput in situ cultivation of "uncultivable" microbial species. *Appl. Environ. Microbiol.*, 76(8), pp. 2445-2450.

Paul, E. A. (2014) *Soil microbiology, ecology and biochemistry*. Academic press.

Penno, C., Hachani, A., Biskri, L., Sansonetti, P., Allaoui, A. and Parsot C. (2006). Transcriptional slippage controls production of type III secretion apparatus components in *Shigella flexneri*. *Mol. Microbiol.*, 62(5), pp. 1460-1468.

Pernthaler, A., Pernthaler, J. and Amann, R. (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl. Environ. Microbiol.,* 68, pp. 3094-3101.

Remus-Emsermann, M. N., Lücker, S., Müller, D. B., Potthoff E., Daims, H. and Vorholt, J. A. (2014). Spatial distribution analyses of natural phyllosphere-colonizing bacteria on *Arabidopsis thaliana* revealed by fluorescence in situ hybridization. *Environ. Microbiol.,* 16, 2329-2340.

Ritz, K. (2007). The plate debate: cultivable communities have no utility in contemporary environmental microbial ecology. *FEMS Microbiol. Ecol.* 60, pp. 358-362.

Schmeisser, C., Steele, H. and Streit W. (2007). Metagenomics, biotechnology with non-culturable microbes, *Appl. Microbiol. Biotechnol.*, 75(5), pp. 955-962.

Schmieder, R., Lim, Y. W. and Edwards, R. (2012). Identification and removal of ribosomal RNA sequences from metatranscriptomes. *Bioinformatics* 28, pp. 433-435.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K., Crous, P. W. and Miller, A. N. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. U.S.A.,* 109(16), pp. 6241-6246.

Scholz, M. B., Lo, C.C. and Chain, P. S. (2012). Next generation sequencing and bioinformatic bottlenecks: the current state of metagenomic data analysis. *Curr. Opin. Biotechnol.*, 23(1), pp. 9-15.

Simon, C. and Daniel, R. (2011). Metagenomic analyses: Past and future trends. *Appl. Environ. Microbiol.,* 77, pp. 1153-1161.

Smith, C. J. and Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol. Ecol.,* 67, pp. 6-20.

Spohn, M., Carminati, A. and Kuzyakov, Y. (2013). Soil zymography–a novel in situ method for mapping distribution of enzyme activity in soil. *Soil Biol. Biochem.,* 58, pp. 275-280.

Spohn, M. and Kuzyakov, Y. (2013). Distribution of microbial-and root-derived phosphatase activities in the rhizosphere depending on P availability and C allocation–coupling soil zymography with ^{14}C imaging. *Soil Biol. Biochem.,* 67, pp. 106-113.

Spohn, M. and Kuzyakov, Y. (2014). Spatial and temporal dynamics of hotspots of enzyme activity in soil as affected by living and dead roots–a soil zymography analysis. *Plant Soil* 379, pp. 67-77.

Spohn, M., Ermak, A. and Kuzyakov, Y. (2013). Microbial gross organic phosphorus mineralization can be stimulated by root exudates–A 33 P isotopic dilution study. *Soil Biol. Biochem.* 65, pp. 254-263.

Spohn, M., Treichel, N. S., Cormann, M., Schloter, M. and Fischer, D. (2015). Distribution of phosphatase activity and various bacterial phyla in the rhizosphere of *Hordeum vulgare* L. depending on P availability. *Soil Biol. Biochem.*, 89, pp. 44-51.

Stewart, F. J., Ottesen, E. A. and DeLong, E. F. (2010). Development and quantitative analyses of a universal rRNA-subtraction protocol for microbial metatranscriptomics. *ISME J.,* 4, pp. 896-907.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W. and Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6, pp. 579-591.

Thomas, T., Gilbert, J. and Meyer, F. (2012). Metagenomics - a guide from sampling to data analysis. *Microb. Informatics Experiment.,* 2, pp. 1.

Torsvik, V. and Øvreås, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.,* 5, pp. 240-245.

Tringe, S. G., von Mering, C., Kobayashi, A., Salamov, A. A., Chen, K., Chang, H. W., Podar, M., Short, J. M., Mathur, E. J., Detter, J. C., Bork, P., Hugenholtz, P. and Rubin, E. M. (2005). Comparative metagenomics of microbial communities. *Science* 308, pp. 554-557.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., Solovyev, V. V., Rubin, E. M., Rokhsar, D. S. and Banfield, J. F. (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428, pp. 37-43.

VanGuilder, H. D., Vrana, K. E. and Freeman, W. M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* 44, pp. 619.

Wagner, L. A., Weiss, R. B., Driscoll, R., Dunn, D. S. and Gesteland R. F. (1990). Transcriptional slippage occurs during elongation at runs of adenine or thymine in *Escherichia coli*. *Nucl. Acid. Res.*, 18(12), pp. 3529-3535.

Walsh, F., and Duffy B. (2013). The culturable soil antibiotic resistome: a community of multi-drug resistant bacteria. *PLoS ONE*, 8(6), pp. e65567.

Watve, M. G., Tickoo, R., Jog, M. M. and Bhole, B. D. (2001). How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.*, 176(5), pp. 386-390.

Wilkinson, D. G. (1998). *In situ hybridization: a practical approach*. Oxford University Press.

Yi, H., Cho, Y. J., Won, S., Lee, J. E., Yu, H. J., Kim, S., Schroth, G. P., Luo, S. and Chun, J. (2011). Duplex-specific nuclease efficiently removes rRNA for prokaryotic RNA-seq. *Nucl. Acid. Res.,* 39, pp. E140.

Appendix 3 Development of marker genes for jasmonic acid signaling in shoots and roots of wheat

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Abstract

The jasmonic acid (JA) signaling pathway plays key roles in a diverse array of plant development, reproduction, and responses to biotic and abiotic stresses. Most of our understanding of the JA signaling pathway derives from the dicot model plant *Arabidopsis thaliana,* while corresponding knowledge in wheat is somewhat limited. In this study, the expression of 41 genes implicated in the JA signaling pathway has been assessed on 10 day-old bread wheat seedlings, 24 h, 48 h, and 72 h after methyl-jasmonate (MeJA) treatment using quantitative real-time PCR. The examined genes have been previously reported to be involved in JA biosynthesis and catabolism, JA perception and signaling, and pathogen defense in wheat shoots and roots. This study provides evidence to suggest that the effect of MeJA treatment is more prominent in shoots than roots of wheat seedlings, and substantial regulation of the JA pathway-dependent defense genes occurs at 72 h after MeJA treatment. Results show that the expression of 22 genes was significantly affected by MeJA treatment in wheat shoots. However, only *PR1.1* and *PR3* were significantly differentially expressed in wheat roots, both at 24 h post-MeJA treatment, with other genes showing large variation in their gene expression in roots. While providing marker genes on JA signaling in wheat, future work may focus on elucidating the regulatory function of JA-modulated transcription factors, some of which have well-studied potential orthologs in *Arabidopsis*.

Key words

Jasmonate; marker genes; PR genes; transcription factor; wheat

Abbreviations

AOS = allene oxide synthase; CHI = chitinase; HPODE = Hydroperoxyoctadeca-9Z, 11E-dienoic acid; JA = jasmonic acid; MeJA = methyl jasmonate; NPR = nonexpressor of pathogenesis-related

(gene); OPDA = oxo phytodienoic acid; OPR = 12-oxo-phytodienoic acid reductase; PAL = phenylalanine ammonia lyase; PR = pathogenesis-related (genes); SA = salicylic acid; Ta = *Triticum aestivum;* WCI = wheat chemically induced (genes); ZAT = Zn transporter (gene).

1. Introduction

Jasmonic acid (JA) is an oxylipin hormone derived from linolenic acid which is crucial for plants to regulate growth and development as well as to respond to biotic and abiotic stresses.[1] The JA pathway has been better characterized in dicot models such as *Arabidopsis* and tobacco and includes JA biosynthesis followed by JA signal transduction, which starts in chloroplasts. Briefly, lipoxygenases (LOXs) which are encoded by *LOX* genes oxygenate the phospholipids of linolenic acid. Linolenic acid is then liberated from membrane lipids and forms hydroperoxy octadecadienoic acid (HPODE). Under the action of an allene oxide synthase (AOS) and an allene oxide cyclase (AOC), respectively, encoded by *AOS* and *AOC* genes, HPODE is converted into 12-OPDA,[2, 3] which is subsequently reduced to JAs via the catalysis of a peroxisome-localized enzyme, 12-oxo-phytodienoic acid reductase 3 (OPR3), followed by three cycles of β-oxidation in the peroxisome.[4, 5] Afterwards, JA-Ile, the JA bioactive form of JA, is formed through a conjugation of JA and isoleucine (Ile) under the action of a GH3 family amido synthetase. JA-Ile is subsequently recognized by CORONATINE INSENSITIVE 1 (COI1)-JASMONATE ZIM DOMAIN (JAZ) co-receptor complexes and activates a signaling cascade for the induction of a series of defense (e.g. *PR*) genes which are also expressed in response to wounding, insect herbivory and necrotrophic pathogens.[6] A schematic presentation for JA biosynthesis and the JA pathway cascade is shown in Fig.1.

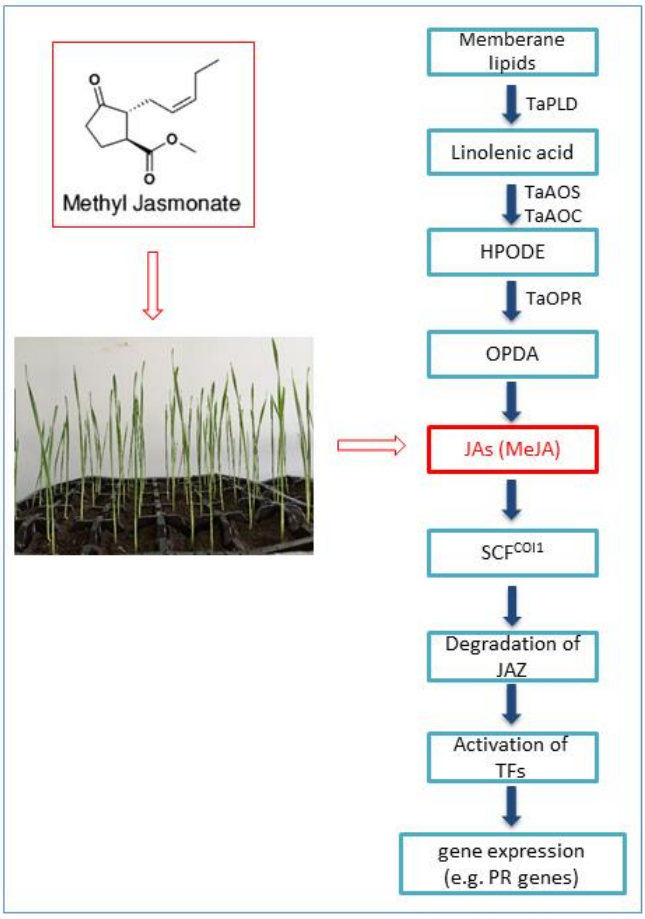

Fig. 1 Schematic diagram of the JA signaling pathway in monocot plants with inclusion of some wheat genes used in this study (adapted from Lyons et al. 2013).

In comparison to *Arabidopsis* and other monocots such as rice and maize, knowledge on JA signaling and biosynthesis in wheat is limited and fragmented.[7] However, JA-dependent responses to diseases, biotic and abiotic stresses have been increasingly investigated in wheat during the last two decades. The *PR* genes *PR1.1* and *PR1.2*, as well as a lipase- and two chitinase- (*CHI1*, *CHI4*) encoding genes were highly induced in 3 weeks post-emergence wheat by JA application. The differential expression of these genes upon JA treatment was also potentiated by common bunt, a disease caused by the closely related fungi *Tilletia tritici* and *Tilletia laevis*.[8] In 2 week-old wheat seedlings, application of JA induced six *PR* genes and four putative defense genes (*TaGLP2a*, *TaPERO*, *WCI2*, *WCI3*), which were also induced by *Fusarium pseudograminearum* infection, the causative agent of wheat crown rot disease.[9] Similarly, using a transcriptome-based method, it was revealed that JA biosynthesis genes such as *LOX*, *AOS*, *AOC* and *OPR3* and JA signaling transduction genes, including *COI1*, *JAZ*, *MYC2,* were induced in a fusarium head blight resistant wheat variety.[10] These findings support the hypothesis that the JA pathway is greatly involved in

the defense against wheat pathogens.[11] The JA signaling pathway also mediates the response to biotic stress from pest attack in wheat. For instance, patterns and levels of genes involved in JA signaling, such as *LOX*, *AOS*, and *AOC*, were differentially affected in wheat in defense response to incompatible interactions with Russian wheat aphids, a serious pest of cereal crops worldwide.[12] Functions which are unrelated to defense have been recently proposed for some genes involved in the JA signaling pathway. For example, overexpression of *TaAOC1* enhanced salinity tolerance in wheat via a JA pathway-dependent manner.[13] Furthermore, wheat genes belonging to the WRKY and MYB transcription factor family have been found to be differentially expressed in wheat under conditions of biotic and abiotic stress, such as *Fusarium graminearum* infection, extreme temperatures (3°C and 40°C), high salinity stress (10% NaCl), osmotic stress and treatment with SA.[14, 15]

However, up-to-date information on plant gene expression during JA signaling in wheat is fragmented and is only presented for either shoots or roots. In this study, we systematically evaluated transcriptional levels of various genes that have been associated with JA signaling in wheat roots and shoots using quantitative real-time PCR. Our findings complement the current knowledge on marker genes for the JA pathway in wheat and will facilitate future studies on this pathway in wheat and other monocot plants.

2 Materials and Methods

2.1 Plant growth conditions, treatments and sampling

A total of 180 wheat seeds (Crusader variety) were planted in a potting mix (Searles, Australia). After stratification at 4°C for 5 days, seeds were transferred to a controlled environment chamber (Percival Scientific, Boone, IA, USA) at 20-24°C with a light intensity of 150 µmol m^{-2} s^{-1}. MeJA was applied for the induction of the JA signaling pathway as follows: 5 µL of neat MeJA was diluted in 995 µL absolute ethanol. As MeJA is a volatile, a volume of 300 µL of the MeJA solution was injected into a cotton ball attached on the lid of the tray. All trays were then immediately wrapped with two tightly sealed transparent plastic bags. Control plants were mock-treated with an equal amount of the solvent ethanol. Each treatment included three biological replicates, and each biological replicate contained a pool of ten plants. During the plant exposure to treatments, tray positions were changed daily in the growth chamber to ensure randomization. To evaluate the transcript abundances of marker genes in response to MeJA treatments, plants were harvested 24 h, 48 h and 72 h after application of the MeJA-treatment. Roots and shoots were stored at -80°C prior to RNA extraction.

2.2 RNA extraction and qRT-PCR analysis

Wheat shoots and roots were ground to a fine powder in liquid nitrogen, and total RNA isolations were performed with the SV Total RNA Isolation Kit (Promega) according to the manufacturer's recommendations. The concentration of the obtained RNA samples was measured using a Nanodrop spectrophotometer (Thermo Scientific). The quality (integrity) of RNA samples was further confirmed by agarose gel electrophoresis (1%). The cDNA was generated by reverse transcription with the Superscript III kit (Life Technologies) from 1.5 µg of total RNA in a 20 µL reaction using both random hexamers and oligo dT primers. Relative quantification of gene expression was performed by using SYBR Green RT-PCR mixtures on a ViiA™ 7 sequence detection system (Applied Biosystems, USA). Targeted genes for quantitative analyses were selected based on previous studies involving JA biosynthesis, signaling and defense-related genes in wheat. Specific primers used in this study were either designed using the Primer Express Software v2.0 (Applied Biosystems) or selected from previous reports. All primers used in this study are listed in Supplementary Table S1. Real-time quantitative reverse transcriptase PCR (qRT-PCR) assays were performed in a 10 µL reaction containing 5 µL SYBR® Green PCR Master Mix, 1 µL of a primer mix (0.3 µM for each primer), and 4 µL of cDNA templates (diluted 30 times from the original cDNA synthesis reaction). 18S rRNA was used as the housekeeping gene for normalization; cDNA for these primers were diluted 500 times prior to PCR reactions. PCR cycling included 95°C for 10 min (heat activation), 40 cycles at 95°C for 15 s, 60°C for 1 min (amplification); and then 95°C for 2 min, 60 °C for 15 s, and 95 °C for 15 s (melt curve analysis). Relative expression of each target gene was investigated using three biological replicates with two technical replicates, each. Data analysis was then performed with ViiA 7 RUO Software (Applied Biosystems) with the 18S rRNA gene as an endogenous reference for normalization.

2.3 Statistical analysis

Means and standard errors were calculated from three biological replicates. Two-tailed t-tests were performed to determine significant differences at the 5% significance level. Heatmaps and bar graphs were generated by the software R-3.2.2 and Graphpad Prism 6.0.1, respectively.

3 Results and Discussion

Ten day-old wheat seedlings were used for the evaluation of potential marker genes of the JA signaling pathway. During wheat sampling, no evidence of a phytotoxic effect induced by MeJA

treatment was observed. Expression levels (transcript abundances) of each gene in shoots and roots after MeJA treatment are summarized in Fig. 2 and Fig. 3, respectively. Corresponding bar graphs displaying relative expression profiles for each gene in both roots and shoots are shown in Supplementary Fig. S1(a)~(v). The following section details the results and discussions of the genes examined.

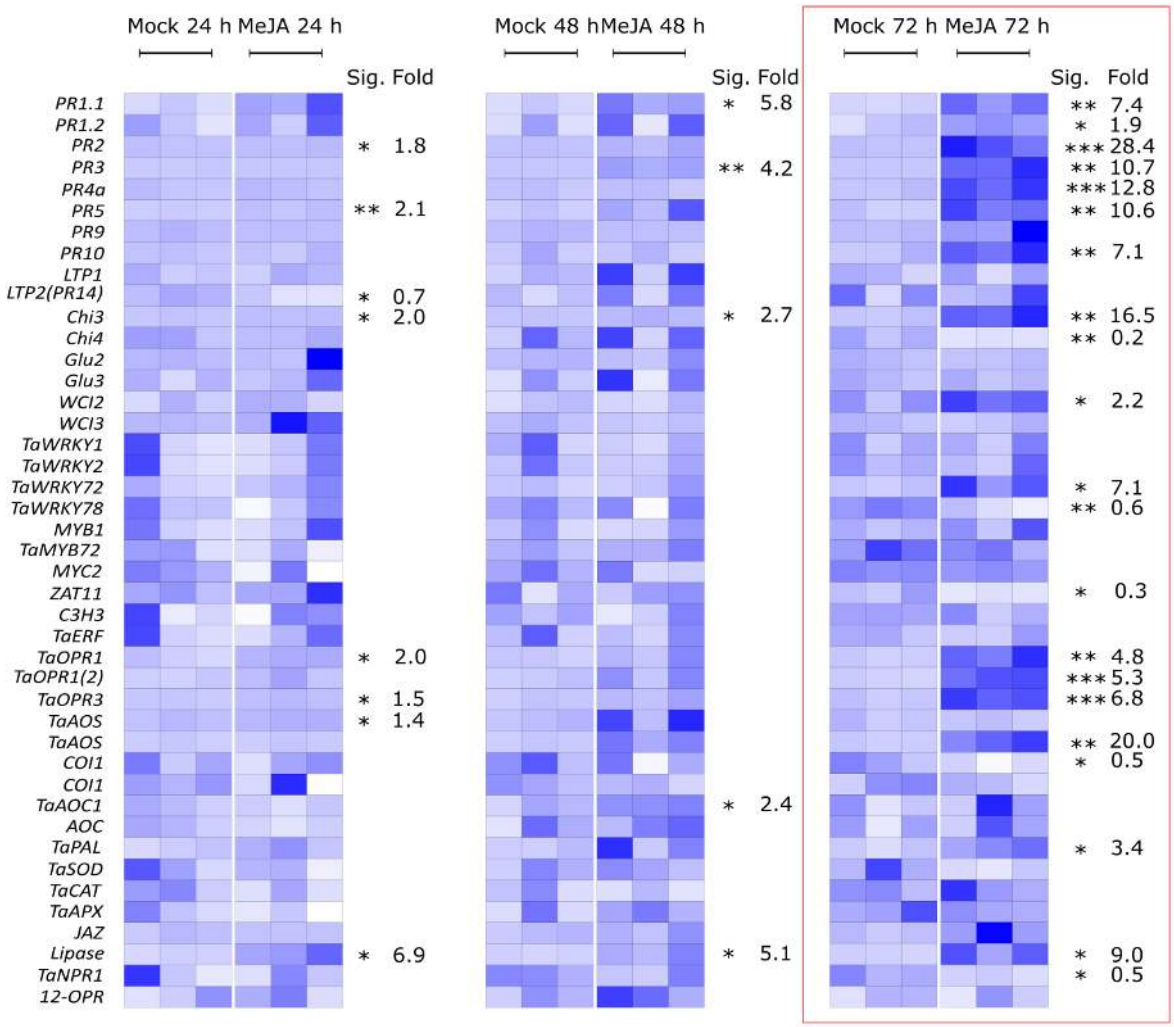

Fig. 2 Heatmap summarizing variation in wheat gene expression between mock and - MeJA-treated shoot samples. Significant differences are indicated by the asterisk(s) after the heatmap blocks of each gene ($P < 0.05$ $^{(*)}$, $P < 0.01$ $^{(**)}$, $P < 0.001$ $^{(***)}$). The numbers in Figure 2 show corresponding fold changes of induced/suppressed genes after MeJA treatment.

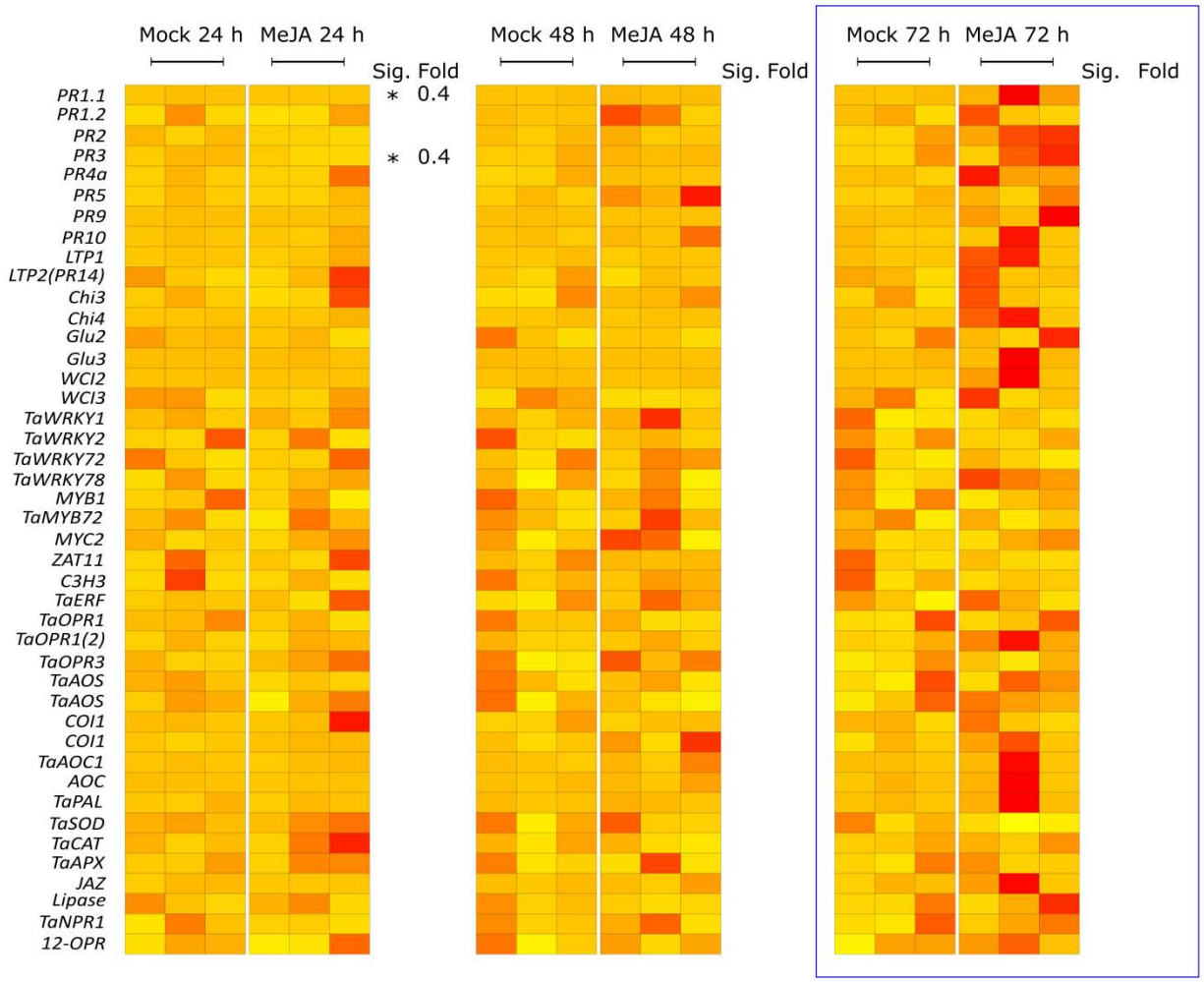

Fig. 3 Heatmap summarizing variation in wheat gene expressions between mock and MeJA-treated root samples. Significant differences are indicated by the asterisks after each gene name ($P < 0.05$ [(*)]). The numbers in Figure 3 show corresponding fold changes of suppressed genes after MeJA treatment.

3.1 JA biosynthesis-associated genes

Four genes whose functions have been associated with JA biosynthesis were chosen for the gene expression analysis, which include genes encoding a wheat allene oxide synthase (*TaAOS*), a wheat allene oxide cyclase (*TaAOC1*), and two wheat oxophytodienoate reductases (*TaOPR1* and *TaOPR3*). *TaAOS* was induced in shoots but only at 24 h after MeJA treatment (Fig.2; Supplementary Fig.S1(q)). During JA biosynthesis, the enzyme allene oxide synthase (AOS) encoded by this gene catalyzes the conversion of hydroperoxyoctadecadienoic acid (HPODE) to 12-oxo-phytodienoic acid (OPDA). In a previous study, *TaAOS* was induced in wheat by *Sitobion avenae* attack.[16] The expression of *TaAOC1* in shoots was potentiated by 2.4-fold at 48 h after MeJA treatment (Fig.2; Supplementary Fig.S1(v)). TaAOC1 and TaAOS both catalyze the first step

of the lipoxygenase pathway, and its encoded enzyme AOC mainly confers the unstable products of AOS with correct enantiomeric structure of natural JA in the α-linolenic acid metabolism pathway.[7] Constitutive expression of this wheat gene in *Arabidopsis thaliana* and bread wheat led to a higher JA content in plants and shorter developed roots along with an enhanced tolerance to salinity.[13]

TaOPR1 (encoding wheat 12-oxo-phytodienoic acid reductase I) was significantly upregulated in shoots by 2.0- and 4.8-fold at 24 h and 72 h after MeJA treatment, respectively (Fig.2; Supplementary Fig.S1(s)). OPRs encoded by *OPR* genes catalyse the production of JA from its precursor of OPDA through the reduction of the double bond.[7] In both *Arabidopsis* and wheat, *OPR1* is involved in biological processes of plant growth and development, and can be stimulated by a variety of environmental and chemical stimuli, such as wounding, pathogen invasion and application of brassinosteroids.[17-19] It has been recently revealed that *TaOPR1* promoted wheat's salinity tolerance capability via increasing ABA signaling and scavenging reactive oxygen species (ROS), without involving the JA signaling pathway.[20] *TaOPR3* expression in shoots was increased by 1.5- and 6.8-fold at 24 h and 72 h after MeJA treatment, respectively (Fig.2; Supplementary Fig.S1(u)). *F. gramineraum* infection induced *TaOPR3* together with another 13 genes associated with JA biosynthesis in the wheat landrace Wangshuibai.[10] In *Arabidopsis*, among the six described *OPRs*, only *OPR3* is involved in JA biosynthesis, which can be induced by touch, wind, UV light, application of detergent, wounding, and brassinosteroids.[21] From what has been discussed above, it is clear that the genes of *TaAOS*, *TaAOC1*, *TaOPR1* and *TaOPR3* were greatly induced in wheat shoots after exogenous treatment with MeJA. Additionally, as these genes are not only essential for the synthesis of JA and its methyl ester but are also involved in plant response to biotic and (or) abiotic stresses, these genes are worth being assessed in future molecular studies on wheat.

3.2 Genes associated with JA signaling

COI1 expression was slightly but significantly repressed by 0.5-fold at 72 h after JA treatment in shoots (Fig.2; Supplementary Fig.S1(i)). In *Arabidopsis* and rice, COI1 forms a functional E3-type ubiquitin ligase complex that targets JAZ proteins (negative regulators for JA signaling) for degradation and *COI1* was not induced after JA treatment .[22] The functions of *COI1* in wheat JA perception and/or signaling are currently unknown. However, in wheat roots, *COI1* was induced within 6 h after inoculation with the *Pseudomonas fluorescens* biocontrol strain Q8r1-96.[23] and our data show that *COI1* was downregulated in the shoots of wheat seedlings (10-day old) by MeJA treatment.

3.3 Transcription factors

Transcription factors are key regulators for the expressions of many *PR* genes in monocot plants.[7] For the five TFs tested in this study, *TaWRKY72a/b*, *TaWRKY78* and *ZAT11* changed in gene expressions after MeJA treatment, which indicates that these genes could be involved in the JA signaling pathway of wheat. Paralogous transcription factors (TFs) of the wheat *WRKY* family, such as *TaWRKY72*a/b, *TaWRKY78* and *ZAT11*, are crucial components in regulating the expression of defense-related genes.[24, 25] The expression of *TaWRKY72*a/b in shoots increased 7.1-fold at 72 h after MeJA treatment (Fig.2; Supplementary Fig.S1(l)). *TaWRKY72*a/b has been shown to be expressed in leaves, roots, and crown and was up-regulated following the maturation and senescence of wheat leaves, which suggests that they may play important roles in regulating wheat leaf senescence.[14] The expression of *TaWRKY78* decreased significantly by 0.6-fold 72 h after MeJA application (Fig.2; Supplementary Fig.S1(m)). It has been demonstrated that *TaWRKY78* and its *Arabidopsis* orthologue, *AtWRKY20* are able to induce the promoter of *wPR4e* (coding for Wheatwin5) and the *wPR4e Arabidopsis* orthologue *AtHEL,* respectively. *TaPR4* genes were induced by treatment with the SA analogue benzothiadiazole (BTH) and MeJA, indicating that *TaWRKY78* is involved in both SA- and JA-dependent defense response pathways.[26] In *Arabidopsis*, WRKYs are most commonly associated with SA signaling, while wheat WRKY TFs examined in the present study have also been strongly influenced by the MeJA treatment (Fig. 2). This has also been reported to be the case in rice.[27]

ZAT11 belongs to the zinc transporter family protein. In wheat shoots, *ZAT11* was significantly downregulated by 0.3-fold at 72 h after MeJA treatment (Fig.2; Supplementary Fig.S1(n)). *ZAT11* (encodes zinc finger-C2H2 type family protein of *Arabidopsis thaliana* 11) is inducible by many stresses and regulates the expression of ascorbate peroxidase, which provides protection against hydrogen peroxide during oxidative stress.[28] ZAT11 is also a dual-function transcriptional regulator that positively regulates primary root growth, but negatively regulates Ni^{2+} tolerance.[29] The function of *ZAT11* gene in wheat is still unknown but is likely to be different as its expression remained unchanged upon treatment with *F. pseudograminearum* CS3096.[30] If *TaWRKY72a/b*, *TaWRKY78* and *ZAT11* are used as JA pathway marker genes in wheat, their involvement in other pathways should also be considered.

3.4 Pathogenesis-related genes

PR1.1 (pathogenesis-related 1 basic) was upregulated in shoots at 48 h and 72 h after MeJA treatment by 5.8- and 7.4-fold, respectively, and was downregulated in roots by 0.6-fold at 24 h post

treatment (Fig.2; Supplementary Fig.S1(a)). Similarly, *PR1.2* (pathogenesis-related 1 neutral) was induced in shoots by 1.9- fold at 72 h after MeJA treatment (Fig.2; Supplementary Fig.S1(b)). These two *PR* genes were also induced in wheat shoots by infection with the fungal pathogen *Erysiphe graminis*.[31] The induction of *PR1* in shoots suggests a cross-talk between the JA and SA signaling pathways, as wheat *PR1* has also been reported to be typically induced during SA pathway activation[7]. For example, the expression of *PR1* was elevated in *F. pseudograminearum*-infected wheat spikes, which was accompanied by an accumulation of SA.[32] In addition, *PR2* (encoding beta-1,3-endoglucanase) was upregulated by 28.4-fold in shoots at 72 h after MeJA treatment (Fig.2; Supplementary Fig.S1 (c)). *PR3* was downregulated in roots by 0.6-fold by MeJA treatment (Fig. 3; Supplementary Fig. S1(d)), but was induced by *Fusarium asiaticum* infection which causes head blight and seedling blight in both wheat spikes and seedlings.[33] Interestingly, *PR2* and *PR3* are reported to be greatly induced in germinating wheat seeds upon infection with the hemibiotroph *Fusarium culmorum*.[34] This pathogen has a short biotrophic stage and then changes to a necrotrophic stage, which is when the JA pathway is usually activated.[35] At 72 h after MeJA treatment, *PR4a* (*wheatwin1-4*) expression in shoots increased by 12.8-fold (Fig.2; Supplementary Fig.S1(e)). The activation of *PR4a* genes has been reported to be involved in both JA and SA-dependent defense response pathways.[26] Besides protecting wheat against fungal pathogens, wheatwin genes were developmentally regulated in the grain and may play a role in response to high temperatures.[36] PR4 proteins show antifungal activity against several phytopathogenic fungi and have been demonstrated to possess ribonucleasic activity correlated to their antifungal capacity.[37, 38] *PR5* encodes a thaumatin-like protein which exhibits antifungal activity against snow mold and *Microdochium nivale*. In shoots, *PR5* (WAS-3a) was induced by 2.1-fold at 72 h post MeJA treatment (Fig.2; Supplementary Fig.S1(f)). *PR5* encodes the major isoform of thaumatin-like protein in winter wheat cells and is markedly induced by treatment with abscisic acid (ABA) and by other elicitors, including chitosan and beta-glucan.[39] *PR10* encodes a ribonuclease-like protein which is a pathogen-induced putative peroxidase from wheat. This gene was significantly induced in shoots by 7.1-fold at 72 h upon MeJA treatment (Fig. 2; Supplementary Fig. S1(g)). This gene is induced by a range of pathogens and plays additional roles in development and enzymatic reactions.[40] *PR14* (*LTP-2*) which codes for a non-specific lipid transfer protein (ns-LTP), decreased by 0.7-fold at 24 h after MeJA treatment (Fig.2; Supplementary Fig.S1(j)). This ns-LTP has previously been isolated by Lu et al. (2005) [41] from a wheat suppression subtractive hybridization (SSH) cDNA library for common bunt (*Tilletia tritici*) infections. LTPs are widely known as ubiquitous proteins that are relevant to plant development and stress responses.[41] Another study demonstrated a significant increase in LTP expression in one week-old seedlings after treatment with MeJA and SA.[8] Collectively this shows that exogenous application of MeJA on wheat leads to

the induction of a diverse range of *PR* genes which provide defensive functions. Prior upregulation of these genes may lead to a higher preparedness of wheat plants to subsequent pathogen attack and should be the subject of further investigation.

3.5 Other important plant defense genes

Besides genes that have previously been recognized as designated orthologs of *PR* genes in wheat, there is a number other potentially important defense genes involved in the wheat JA pathway. These include, for instance chitinase and lipase encoding genes, and those genes involved in the cross-talk with other signaling pathways, e.g. *TaNPR1,* linking JA and SA signaling. Chitinases are pathogenesis-related proteins that hydrolyze chitin, an essential structural component of fungal cell walls. *CHI3* expression was significantly increased in shoots at 24 h, 48 h and 72 h after MeJA treatment by 2.0-, 2.7- and 16.5-fold, respectively (Fig.2; Supplementary Fig.S1(o)). *CHI3* has been shown to be induced by *F.graminearum*.[42] In shoots, the expression of the *CHI4 precursor* was downregulated 72 h post MeJA treatment by 0.2-fold (Fig.2; Supplementary Fig.S1(p)). This gene has been previously reported to be induced by both MeJA and common bunt infections (*T. tritici*) in wheat seedlings.[8] *WCI2* was significantly induced in shoots at 72 h post MeJA treatment by 2.2-fold (Fig.2; Supplementary Fig.S1(k)). *WCI* genes were involved in systemic acquired resistance (SAR), and a specific set of *WCI* genes have been induced by BTH. Induction of WCI genes was involved in increased wheat resistance to powdery mildew infection through affecting multiple steps of pathogen development.[43] The *LIPASE* gene was isolated from a wheat SSH cDNA library for common bunt infections by Lu et al. (2005) [41] and was significantly induced in shoots 24 h, 48 h and 72 h post MeJA treatment (Fig.2; Supplementary Fig.S1(h)). The encoded lipases have been associated with pathogen resistance responses in plants through the SA signaling pathway but it also was reported to be strongly upregulated by MeJA in two and three week-old wheat seedlings.[8]

TaNPR1 (non-expressor of pathogenesis-related genes) is a key regulator of the SA signaling pathway, and as expected was found significantly repressed by 0.5-fold at 72 h post MeJA treatment (Fig.2; Supplementary Fig.S1(r)). *AtNPR1* plays a center role in the onset of SA-mediated SAR.[44] Importantly, *AtNPR1* is involved in the cross-talk between SA- and JA-dependent pathways and assumes the key role in the suppression of JA-mediated defense responses by the SA pathway.[45-47] In wheat, *TaNPR1* in an HvSGT1-over-expressing line was greatly downregulated at 24 h post inoculation with biotrophic pathogen *Blumeria graminis DC. f. sp. tritici* compared to wild-type.[48] The transcript abundance of *TaPAL* increased by 3.4-fold in shoots at 72 h after MeJA

treatment (Fig.2; Supplementary Fig.S1(t)). Phenylalanine ammonia lyase (PAL) is involved in both SA and JA-dependent pathways and is essential for biosynthesis of phenylpropanoids. PAL is also associated with a variety of functions, including plant host defense against pathogens and response to abiotic stress like wounding.[16, 49] These genes as stated above may provide useful information for future studies on the effects of plant hormone or pathogen treatments on wheat. Their co-regulation by other defense pathways should be noted when used as marker genes for JA signaling in wheat.

3.6 Genes that were not differentially expressed by MeJA

Genes that are related to ROS production/scavenging systems, including *CAT* (catalase), *SOD* (superoxide dismutase) and *APX* (ascorbate-peroxidase), were not affected by MeJA treatment. The Jasmonate ZIM domain (*JAZ*) encoding gene did not respond to MeJA treatment within the examined period of time. Initially observed to be early up-regulated by wounding or JA treatment, JAZ proteins are recognized as targets of the SCFCOI1 complex. The degradation of JAZ allows the release of positively acting TFs, such as *MYC2* (encoding a basic-helix-loop-helix (bHLH) TF) and its homologue MYC3 that bind to JA-responsive elements occurring in promoters of JA-responsive genes via the mediator subunit MED25.[50] *MYC2* has emerged as a master regulator of most aspects of the jasmonate (JA) signaling pathway in *Arabidopsis.*[7] However, a putative *MYC2* ortholog in wheat was not induced in the current study.[10] Additionally a *C3H* encoding C3H-type zinc finger TF, *TaERF* (wheat ethylene-responsive factor-like transcription factor), *TaWRKY1*, *TaWRKY2*, *WCI3* (wheat chemical induced gene 3), *Glu2* (neutral β-1,3; β-1,4-glucanase), *Glu3* (basic β-1,4-glucanase) were not induced within 24-72 hours. It is possible that these genes may have responded to MeJA treatment at even earlier times than 24 h.

3.7 Late response JA signaling genes

In the present study, we found that most -induced genes increased greatly in expression at 72 h-post MeJA treatment, especially those genes involved in plant defence (e.g. designated *PR* genes*, CHI3*, *TaPAL*). This result seems to contradict the notion that plants should respond to pest and pathogen invasions at an earlier stage than 72 h. JA marker gene expression studies in rice have also focused on early responses.[22] However, chemical treatment with MeJA is different from pathogen and pest attacks and it has been reported that phase changes occurring during plant development can determine to what extent a plant responds to different signaling compounds (e.g. MeJA and SA). Defense-related genes of 1-, 2- and 3-week stage seedlings responded differentially to SA and MeJA treatment. Spraying MeJA solution on wheat shoots greatly induced *CHI1*, *CHI3*, *CHI4*, *PR1.1* and

Lipase genes 24 h after MeJA treatment on three-week old wheat seedlings but not in one-week or two-week old wheat seedlings.[8] In our study, as 10-day old wheat seedlings were used, the early stage of these plants may have potentially led to the late responses of wheat plants to MeJA treatment. Additionally, incubation of wheat with MeJA vapors is different from the spraying method as previously reported.[8,9,11,27,30] In comparison with the spraying method, incubating wheat with MeJA vapor of a relative low concentration (0.025 μL per liter) may have caused a delay of MeJA to reach wheat plants. Therefore, the treatment method used may have also contributed to the late response of wheat to MeJA treatment.

3.8 Large variances in gene expressions among replicates found in wheat roots

Many studies found differential expression in roots during plant defense signaling to be much less pronounced than in shoots.[51] There are several trends that can be seen from the root heatmap data with only a few significantly differentially expressed genes (Fig.3). These may serve as a basis for further studies, taking into consideration the factors that may contribute to larger variation in root samples. In comparison, the variance among shoot samples was much smaller than for root samples (Fig.2; Fig.3). Although not significant, there was a trend of an increase in expression of *PR* genes 72 h-post MeJA treatment (Fig.3), which is consistent with the induced gene expression in shoots. As cDNA synthesis and RT-PCR were implemented in the same batch for root and shoot samples, we assumed that this experiment had been performed technically well. Nevertheless, traces of humic acid or other reverse transcriptase or PCR inhibiting compounds may have been present in root samples. We incubated wheat seedlings with MeJA vapors, which has possibly led to plant roots unevenly accessing MeJA molecules, considering that MeJA vapors first need to penetrate into the soil to reach the roots. In contrast, wheat shoots were evenly exposed to this volatile signaling compound.

4 Conclusions

In this study, using gene expression profiling by qRT-PCR, the expression of the genes potentially involved in JA biosynthetic and signaling pathway was examined at three time points post-MeJA treatment in wheat seedlings. Our results suggest that differential expression of JA-associated genes was more prominent at 72 h after MeJA application. These genes may serve as useful markers to further elucidate JA signaling in wheat or to confer resistance to pests and diseases. For example, the overexpression of JA-modulated *PR* genes may provide resistance against wheat pathogens. The observed differential expression of regulatory genes (including TFs) suggests a regulatory function during JA signaling. These genes may provide powerful tools for modulating JA

signaling in wheat (e.g. towards disease resistance), as this has been highly successful for putative orthologs of these genes in *Arabidopsis*. While most of the present knowledge of the JA signaling pathway derives from the dicotyledonous model plant Arabidopsis thaliana, this study supports the notion that JA signaling in monocotyledonous plants could be used for similar functions.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Creelman RA, Mullet JE. Biosynthesis and action of jasmonates in plants. Annu. Rev. Plant Biol. 1997; 48:355-81.
2. Laudert D, Pfannschmidt U, Lottspeich F, Holländer-Czytko H, Weiler E. Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol. Biol. 1996; 31:323-35.
3. Stenzel I, Hause B, Miersch O, Kurz T, Maucher H, Weichert H, et al. Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol. Biol. 2003; 51:895-911.
4. Stintzi A. The Arabidopsis male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc. Natl. Acad. Sci. USA 2000; 97:10625-30.
5. Strassner J, Schaller F, Frick UB, Howe GA, Weiler EW, Amrhein N, et al. Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J. 2002; 32:585-601.
6. Cheong JJ, Choi YD. Methyl jasmonate as a vital substance in plants. TIG 2003; 19:409-13.
7. Lyons R, Manners JM, Kazan K. Jasmonate biosynthesis and signaling in monocots: a comparative overview. Plant Cell Rep. 2013; 32:815-27.
8. Lu ZX, Gaudet D, Puchalski B, Despins T, Frick M, Laroche A. Inducers of resistance reduce common bunt infection in wheat seedlings while differentially regulating defence-gene expression. Physiol. Mol. Plant Pathol. 2006; 67:138-48.
9. Desmond OJ, Edgar CI, Manners JM, Maclean DJ, Schenk PM, Kazan K. Methyl jasmonate induced gene expression in wheat delays symptom development by the crown rot pathogen *Fusarium pseudograminearum*. Physiol. Mol. Plant Pathol. 2006; 67:171-9.
10. Xiao J, Jin X, Jia X, Wang H, Cao A, Zhao W, et al. Transcriptome-based discovery of pathways and genes related to resistance against Fusarium head blight in wheat landrace Wangshuibai. BMC genomics 2013; 14:197.
11. Li G, Yen Y. Jasmonate and ethylene signaling pathway may mediate Fusarium head blight resistance in wheat. Crop Sci 2008; 48:1888-96.
12. Liu X, Meng J, Starkey S, Smith C. Wheat gene expression is differentially affected by a virulent Russian wheat aphid biotype. J Chem. Ecol. 2011; 37:472-82.
13. Zhao Y, Dong W, Zhang N, Ai X, Wang M, Huang Z, et al. A wheat allene oxide cyclase gene enhances salinity tolerance via jasmonate signaling. Plant Physiol. 2014; 164: 1068-76.
14. Wu H, Ni Z, Yao Y, Guo G, Sun Q. Cloning and expression profiles of 15 genes encoding WRKY transcription factor in wheat (*Triticum aestivem* L.). Prog. Nat. Sci. 2008; 18:697-705.
15. Zhu X, Liu S, Meng C, Qin L, Kong L, Xia G. WRKY transcription factors in wheat and their induction by biotic and abiotic stress. Plant Mol. Biol. Rep. 2013; 31:1053-67.
16. Zhao LY, Chen JL, Cheng DF, Sun JR, Liu Y, Tian Z. Biochemical and molecular characterizations of *Sitobion avenae*-induced wheat defense responses. Crop Prot. 2009; 28:435-42.
17. Reymond P, Weber H, Damond M, Farmer EE. Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell 2000; 12:707-19.
18. Schaller F. Enzymes of the biosynthesis of octadecanoid ‐ derived signalling molecules. J. Exp. Bot. 2001; 52:11-23.
19. Tan CT, Carver BF, Chen MS, Gu YQ, Yan LL. Genetic association of OPR genes with resistance to Hessian fly in hexaploid wheat. BMC Genomics 2013; 14:369.
20. Dong W, Wang M, Xu F, Quan T, Peng K, Xiao L, et al. Wheat oxophytodienoate reductase gene *TaOPR1* confers salinity tolerance via enhancement of abscisic acid signaling and reactive oxygen species scavenging. Plant Physiol. 2013; 161:1217-28.

21. Schaller F, Biesgen C, Müssig C, Altmann T, Weiler EW. 12-Oxophytodienoate reductase 3 (OPR3) is the isoenzyme involved in jasmonate biosynthesis. Planta 2000; 210:979-84.
22. Lee HY, Seo JS, Cho JH, Jung H, Kim JK, Lee JS, Rhee S,Do Choi Y. *Oryza sativa* COI homologues restore jasmonate signal transduction in *Arabidopsis coi1-1* mutants. PLoS ONE 2013; 8:p.e52802.
23. Okubara PA, Call DR, Kwak Y-s, Skinner DZ. Induction of defense gene homologues in wheat roots during interactions with *Pseudomonas fluorescens*. Biol. Control 2010; 55:118-25.
24. Riechmann JL, Meyerowitz EM. The AP2/EREBP family of plant transcription factors. J Biol. Chem. 1998; 379:633-46.
25. Zhao J, Buchwaldt L, Rimmer SR, Sharpe A, McGregor L, Bekkaoui D, et al. Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum*. Mol. Plant Pathol. 2009; 10:635-49.
26. Bertini L, Leonardi L, Caporale C, Tucci M, Cascone N, Di Berardino I, et al. Pathogen-responsive wheat *PR4* genes are induced by activators of systemic acquired resistance and wounding. Plant Sci. 2003; 164:1067-78.
27. Qiu D, Xiao J, Ding X, Xiong M, Cai M, Cao Y, et al. OsWRKY13 mediates rice disease resistance by regulating defense-related genes in salicylate- and jasmonate-dependent signaling. MPMI 2007; 20:492-9.
28. Rizhsky L, Davletova S, Liang H, Mittler R. The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in Arabidopsis. J. Biol. Chem. 2004; 279:11736-43.
29. Liu XM, An J, Han H, Kim S, Lim C, Yun D-J, et al. ZAT11, a zinc finger transcription factor, is a negative regulator of nickel ion tolerance in Arabidopsis. Plant Cell Rep. 2014:1-7.
30. Desmond O. Wheat defence responses induced by the fungal pathogen *Fusarium pseudograminearum*. PhD Thesis, accessed on 12, December, 2014. http://espace.library.uq.edu.au/view/UQ:155236. 2008.
31. Molina A, Görlach J, Volrath S, Ryals J. Wheat genes encoding two types of PR-1 proteins are pathogen inducible, but do not respond to activators of systemic acquired resistance. Mol. Plant Microb. Interac. 1999; 12:53-8.
32. Makandar R, Nalam VJ, Lee H, Trick HN, Dong Y, Shah J. Salicylic acid regulates basal resistance to Fusarium head blight in wheat. Mol. Plant Microb. Interac. 2012; 25:431-9.
33. Li X, Zhang J, Song B, Li H, Xu H, Qu B, et al. Resistance to Fusarium head blight and seedling blight in wheat is associated with activation of a cytochrome P450 gene. Phytopathology 2010; 100:183-91.
34. Caruso C, Chilosi G, Caporale C, Leonardi L, Bertini L, Magro P, et al. Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum*. Plant Sci. 1999; 140:87-97.
35. Bushnell W, Hazen B, Pritsch C, Leonard K. Histology and physiology of Fusarium head blight. Fusarium head blight of wheat and barley 2003:44-83.
36. Altenbach SB, Kothari KM, Tanaka CK, Hurkman WJ. Genes encoding the PR-4 protein wheatwin are developmentally regulated in wheat grains and respond to high temperatures during grainfill. Plant Sci. 2007; 173:135-43.
37. Caporale C, Di Berardino I, Leonardi L, Bertini L, Cascone A, Buonocore V, et al. Wheat pathogenesis-related proteins of class 4 have ribonuclease activity. FEBS Letters 2004; 575:71-6.
38. Bertini L, Caporale C, Testa M, Proietti S, Caruso C. Structural basis of the antifungal activity of wheat PR4 proteins. FEBS Letters 2009; 583:2865-71.
39. Kuwabara C, Takezawa D, Shimada T, Hamada T, Fujikawa S, Arakawa K. Abscisic acid- and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mould, *Microdochium nivale*. Physiol. Plant. 2002; 115:101-10.
40. Liu JJ, Ekramoddoullah AK. The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. Physiol. Mol.

Plant Path. 2006; 68:3-13.
41. Wasternack C. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. 2007; 100:681-97.
42. Kong L, Anderson JM, Ohm HW. Induction of wheat defense and stress-related genes in response to *Fusarium graminearum*. Genome 2005; 48:29-40.
43. Görlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel K-H, et al. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 1996; 8:629-43.
44. Koornneef A, Pieterse CM. Cross talk in defense signaling. Plant Physiol. 2008; 146:839-44.
45. Dong X. NPR1, all things considered. Curr. Opin. Plant Biol. 2004; 7:547-52.
46. Pieterse CM, Van Loon L. NPR1: the spider in the web of induced resistance signaling pathways. Curr. Opin. Plant Biol. 2004; 7:456-64.
47. Spoel SH, Koornneef A, Claessens SM, Korzelius JP, Van Pelt JA, Mueller MJ, et al. NPR1 modulates cross-talk between salicylate-and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 2003; 15:760-70.
48. Xing L, Qian C, Cao A, Li Y, Jiang Z, Li M, et al. The *Hv-SGT1* gene from *Haynaldia villosa* contributes to resistances towards both biotrophic and hemi-biotrophic pathogens in common wheat (*Triticum aestivum* L.). PLoS ONE 2013; 8:e72571.
49. Moran PJ, Thompson GA. Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiol. 2001; 125:1074-85.
50. Kidd BN, Edgar CI, Kumar KK, Aitken EA, Schenk PM, Manners JM, Kazan K. The Mediator complex subunit, PFT1, is a key regulator of jasmonate-dependent defense in *Arabidopsis*. Plant Cell 2009; 21:2237-52.
51. Edgar CI, McGrath KC, Dombrecht B, Manners JM, Maclean DC, Schenk PM, Kazan K. Salicylic acid mediates resistance to the vascular wilt pathogen *Fusarium oxysporum* in the model host *Arabidopsis thaliana*. Australasian Plant Pathol. 2006; 35:581-91.

Supplementary materials for this this study

Table S1 Sequences of primers used to measure wheat gene expression by qRT- PCR

Clone	Gene	Forward	Reverse	Gene description	References
AF159369	*18S*	CAAAGCAAGCCTAC GCTCT	ATACGAATGCCCC CGACT	*Haematococcus pluvialis* 18S ribosomal RNA gene	—
AJ007348	*PR1.1*	CTGGAGCACGAAGC TGCAG	CGAGTGCTGGAGC TTGCAGT	*PR-1* (basic), pathogenesis-related protein 1	(Desmond, et al. 2006)
AJ007349	*PR1.2*	CGTGTGTTTATGTTT GTGTGGTTTG	CCACAGAGCCGCA TGGAT	*PR-1* (neutral), pathogenesis-related protein 1	—
Y18212	*PR2*	CTCGACATCGGTAA CGACCAG	GCGGCGATGTACT TGATGTTC	beta-1,3-endoglucanase	(Desmond, et al. 2006)
AB029934	*PR3*	AGAGATAAGCAAGG CCACGTC	GGTTGCTCACCAG GTCCTTC	*Chi1* gene	—

AJ006098	*PR4a*	CGAGGATCGTGGAC CAGTG	GTCGACGAACTGG TAGTTGACG	*wheatwin 1-2* gene	(Desmond, et al. 2006)
AF442967	*PR5*	ACAGCTACGCCAAG GACGAC	CGCGTCCTAATCT AAGGGCAG	WAS3a thaumatin-like protein	(Desmond, et al. 2006)
X56011	*PR9*	GAGATTCCACAGAT GCAAACGAG	GGAGGCCCTTGTT TCTGAATG	wheat peroxidase	(Desmond, et al. 2006)
CA684431	*PR10*	TTAAACCAGCACGA GAAACATCAG	ATCCTCCCTCGATT ATTCTCACG	ribonucleases, bet v 1-related proteins	(Desmond, et al. 2006)
TaBs108F7b	*LTP1*	ACGTAGGTACTCCT CTCGCTGT	GTTGATCGACCAC TTCTTCTCA	wheat lipid transfer protein	(Lu, et al. 2006)
TaBs112C7	*LTP2(PR14)*	GGTCACACACACAC ACACACA	CGGGAGAGAAGT AACAACCAA	wheat nonspecific lipid transfer proteins (ns-LTPs)	(Lu, et al. 2006)
AB029936	*CHI3*	GACCTCCTTGGCGT CAGCTA	TGCATGTCTTCTC GCATCATATAGTC	class 1b neutral chitinase	—
AF112966	*CHI4*	AACGTCGACCCAGG GAACA	AGCAGTAGGAGCA TCGCTAGAAAG	class 4 acidic chitinase	—
Z22874	*Glu2*	CATGGCTAACATCTA CCCGTACCT	GAAGAGCGCGTAG CTCATGTC	neutral β-1,3; β-1,4-glucanase	—
AY091512	*Glu3*	GTACTTCGCCACGG GAAACA	TGGGCTGCCAATC CAGAAC	basic β-1,4-glucanase	—
U32428	*WCI2*	TAGGAACTGGAACT TCACCGAGC	GGTAGTCCTTGAT GTGCAGCGAC	wheat chemically induced (WCI) gene, Lipoxygenase (Fragment)	(Desmond, et al. 2006)
U32429	*WCI3*	AAAGTTGGTCTTGC CACTGACTG	TCGACAAAGCACT TCTGGATTTC	wheat chemically induced (*WCI*) gene, sulfur-rich/thionin-like protein	(Desmond, et al. 2006)
TC207269	*TaWRKY1*	TAATCCCCACATCA GAAGACACTG	TTTGAGGTTTTGA CGGAGGC	transcription factor genes (*TFs*)	(Desmond 2008)
TC199398	*TaWRKY2*	GCAGAGACCCCAGT CGATGA	GGTGCGTGCAGCG GTAGTAT	transcription factor genes (*TFs*)	(Desmond 2008)
CN009320	*TaWRKY72a/b*	ACAACTTCCCCAGG AGCTACTACC	CCTCGTATGTCGTT ACCACCACA	transcription factor genes (*TFs*)	(Desmond 2008)
HM013818	*TaWRKY78*	GATGCAATCCATGG CTTCGA	CATGCGGCCAGCA GAGTTT	transcription factor genes (*TFs*)	—
TC221263	*MYB*[1]	GCAACTTCACCAGC GAGGAG	TGTGCCAGACGTT CTTGATCTC	transcription factor genes (*TFs*)	(Desmond 2008)
JF951955.1	*TaMYB*	CGACGTGTGCTCCA	CGGTGCCATCTGG	transcription factor genes	(Zhang,

	72	TCAA	AGTAG	(*TFs*)	et al. 2012)
unigene15470	*MYC2*	CCGGGGAAAACACCTAAAAT	TGCTCCAGGCTCTCTTTCTC	a basic helix-loop-helix transcription factor	(Xiao, et al. 2013)
TC198896	*ZAT*11	GATGAGTGCCTGGAAGACATACC	CCGAAGCCACCAAATTAAGC	Zn transporter protein	(Desmond 2008)
TC218601	*C3H*[3]	CCACCAAAACAGACTCCCCA	CGTTGATGTCCTCCGTCTGG	transcription factor genes (*TFs*)	(Desmond 2008)
GU452719	*TaERF*	CCTTCTTCTGTTCTGGTCCTCTTG	CTCTGTTCTCGGCGGAAACAC	wheat ethylene-responsive factor-like transcription factor	—
JQ409278	*TaOPR1*	TCGCCCTTCATGGACTACATG	TAGAGGATGCCGTGGTCGTT	wheat oxophytodienoate reductase-1	(Dong, et al. 2013)
unigene 139032	*TaOPR3*	GGAACCACGGATGGTGAATA	GGCAAGTCTGGATTGGACAG	wheat oxophytodienoate reductase-3	(Xiao, et al. 2013)
CA650490	*OPR*	GGAAGGCAACAAAGTGGTG	GGAAGGCAACAAAGTGGTG	12-oxo-phytodienoic acid reductases	(Liu, et al. 2011)
AY196004	*TaAOS*	TCCCGAGAGCGCTGTTTAAA	GACGATTGACGGCTGCTATGA	*Triticum aestivum* allene oxide synthase	—
HM447645	*COI1*	CATTGTGCGAGTGAACTGTGACA	CGCGGAAACCAGACAAGCT	*Triticum aestivum* coronatine insensitive 1-like protein	—
unigene29682	*COI1*	CCTTTGGCAAGAACCGTATC	ATCAAAGCACGGAGCAACTT	*Triticum aestivum* coronatine insensitive 1-like protein	(Xiao, et al. 2013)
KF573524.1	*TaAOC 1*	CGTCTTCGAGGGCGTCTACG	GCAGGTCGGGGATGCCCTTGA	involved in the α-linolenic acid metabolism pathway	(Zhao, et al. 2013)
BJ241555	*AOC*	ATTCATTCAACACTGGTACAAGG	ATCTATTATTGCTCCTGCTAGTAG	allene oxide cyclase	(Liu, et al. 2011)
BT0089921	*LOX*	TGTTGATAGACTGGTGCTGTG	TGAGGATTAACGCTTAGGATCG	Lipoxygenase	(Liu, et al. 2011)
TC294834	*TaPAL*	CGTCAAGAGCTGTGTGAAGATGG	GGTAGTTGGAGCTGCAAGGGTC	phenylalanine ammonia lyase	(Zhang, et al. 2011)
U69632.1	*TaSOD*	CGATAGCCAGATTCCTTTGACT	GAAACCAGCGACCTACAACG	superoxide dismutase	(Zhang, et al. 2011)
X94352	*TaCAT*	TGCCTGTGTTTTTTATCCGAGA	CTGCTGATTAAGGTGTAGGTGTTGA	Catalase	(Zhang, et al. 2011)
TC369354	*TaAPX*	GGTTTGAGTGACCA	GCATCCTCATCCG	ascorbate-peroxidase	(Zhang,

		GGACATTG	CAGCAT		et al. 2011)
unigene5157 3	*JAZ*	CCGTAGCACGGTCT TACCAT	ATATGAGGCGAGC AACTTGG	jasmonate zim-domain proteins 1	(Xiao, et al. 2013)
TaBs117A2	*LIPASE*	CACAAAATATCGAC CCACCAC	ACTGGGTATTCGT CTGTCAGC	wheat lipase	(Lu, et al. 2006)
AX049430	*TaNPR1*	TGAGGGAAGTCGAT CTGAATGAG	GCCCAGTTCCACT GTTTTCACT	wheat nonexpressor of pathogenesis-related Genes 1	—

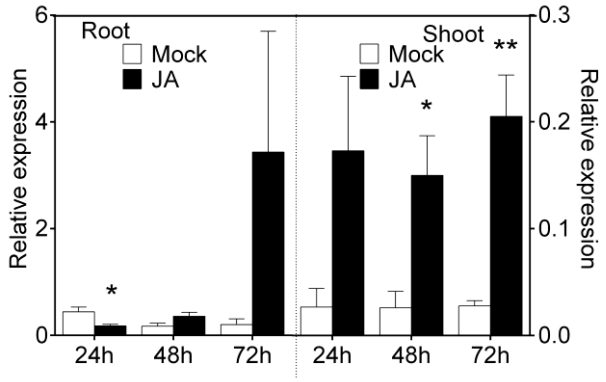
Primers designed in this study were marked with '—';

[1] The *Arabidopsis* Genbank accession number is At1g56160, *Arabidopsis* description: *AtMYB72* (Myb domain protein 72);

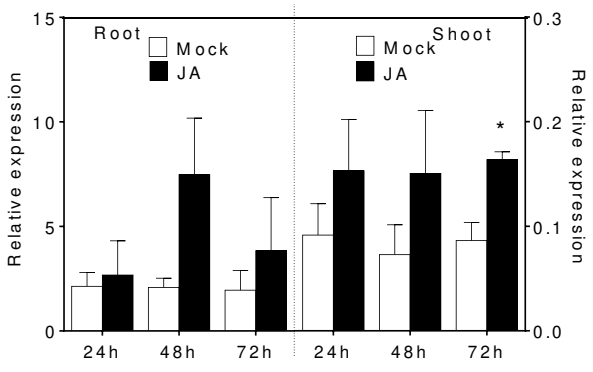
[2] *Arabidopsis* accession At2g37430, *Arabidopsis* description: zinc finger (C2H2 type) family protein;

[3] *Arabidopsis* accession At3g55980, *Arabidopsis* description: zinc finger (C3H type) family protein.

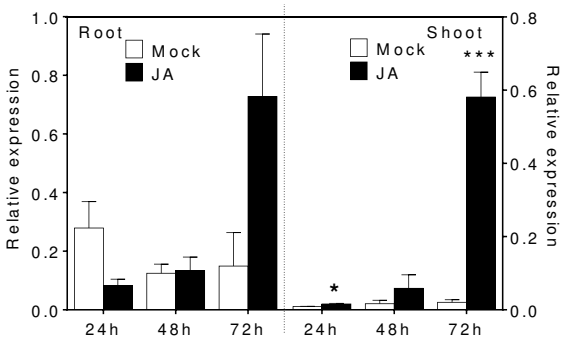
Supplementary Figure S1(a)~(v)



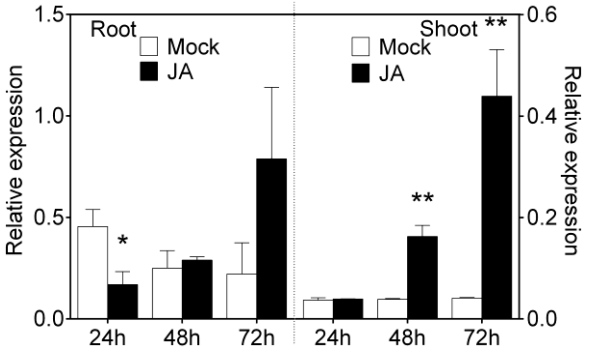
(a) *PR1.1*



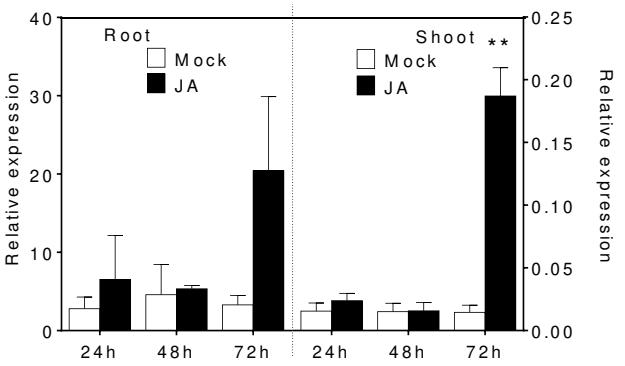
(b) *PR1.2*



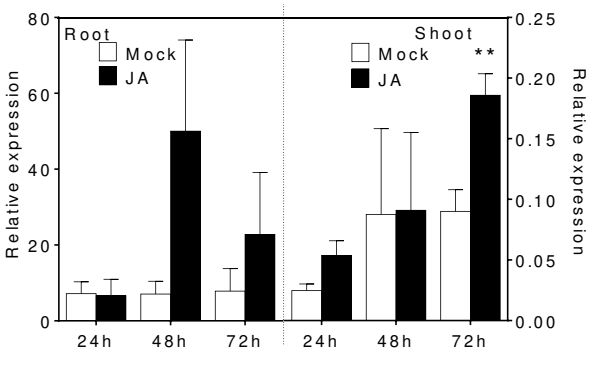
(c) *PR2*



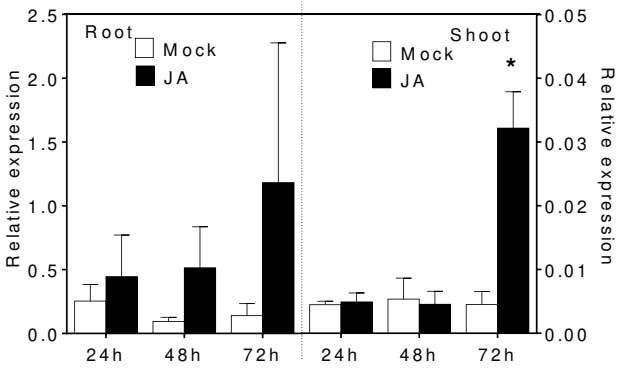
(d) *PR3*(*CHI1*)



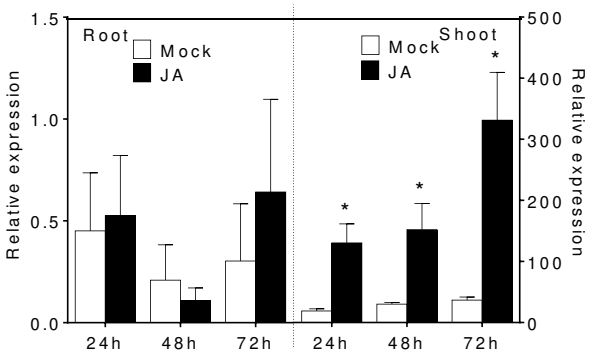
(e) *PR4a* (*Wheatwin 1-2*)



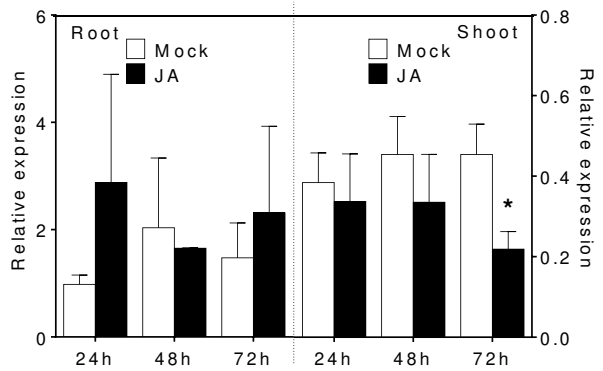
(f) *PR5* (*WAS3a*)



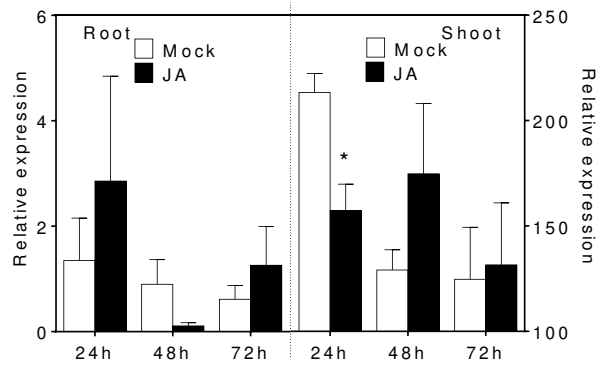
(g) *PR10*



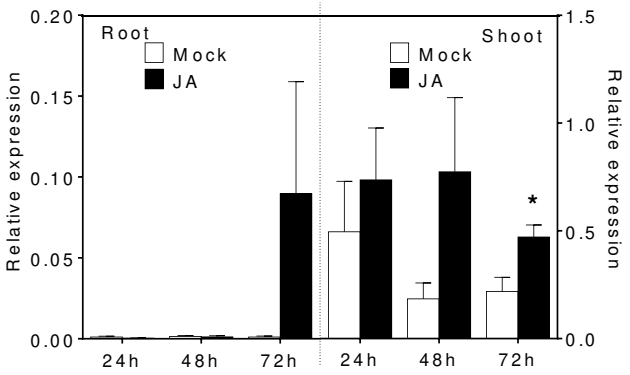
(h) *Lipase*



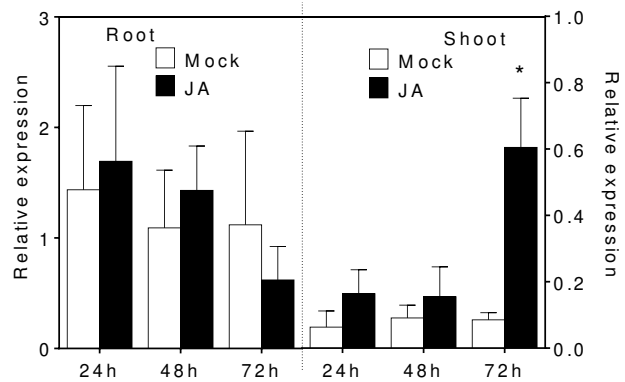
(i) *COI1*



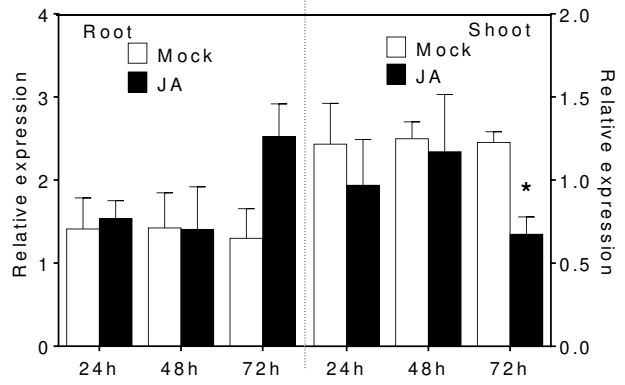
(j) *LTP2*



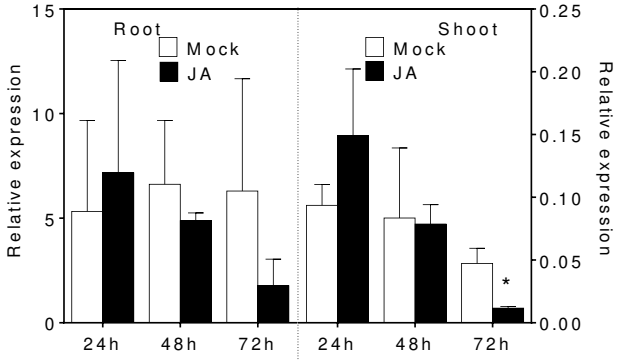
(k) *WCI2*



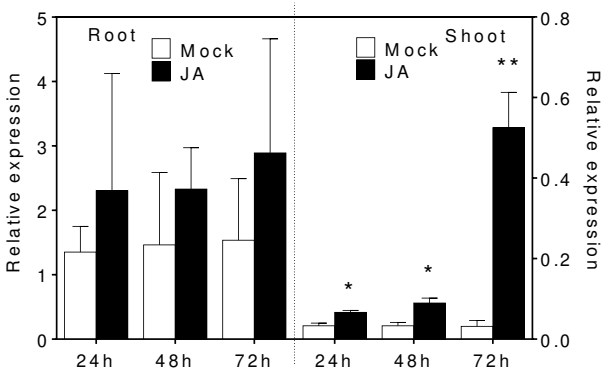
(l) *WRKY72*



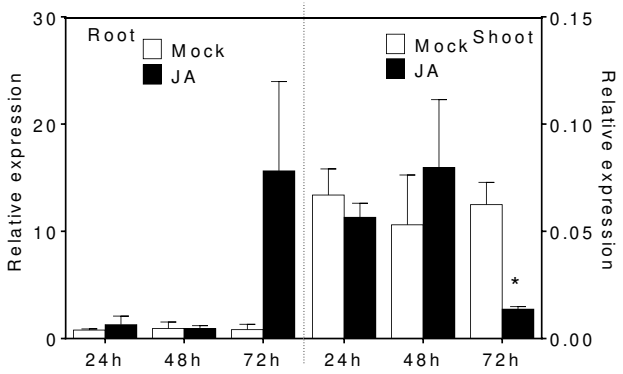
(m) *WRKY78*



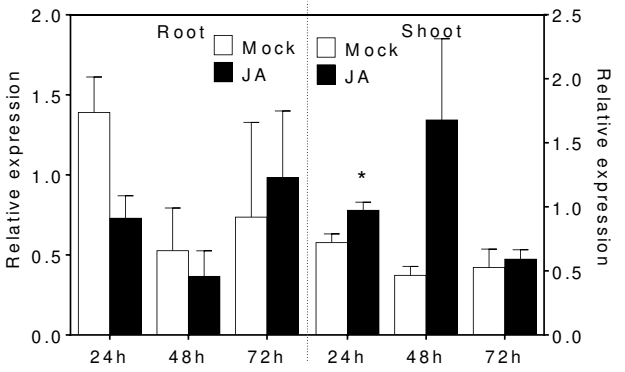
(n) *ZAT*



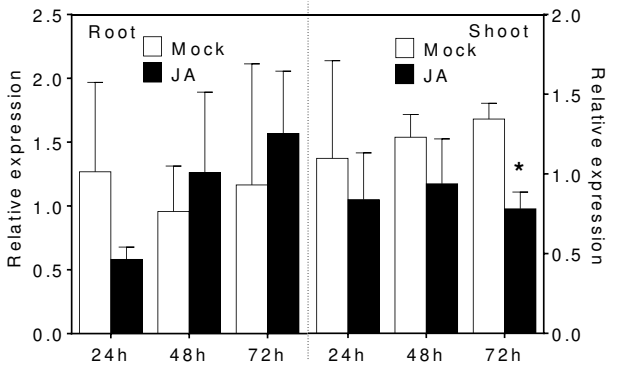
(o) *CHI3*



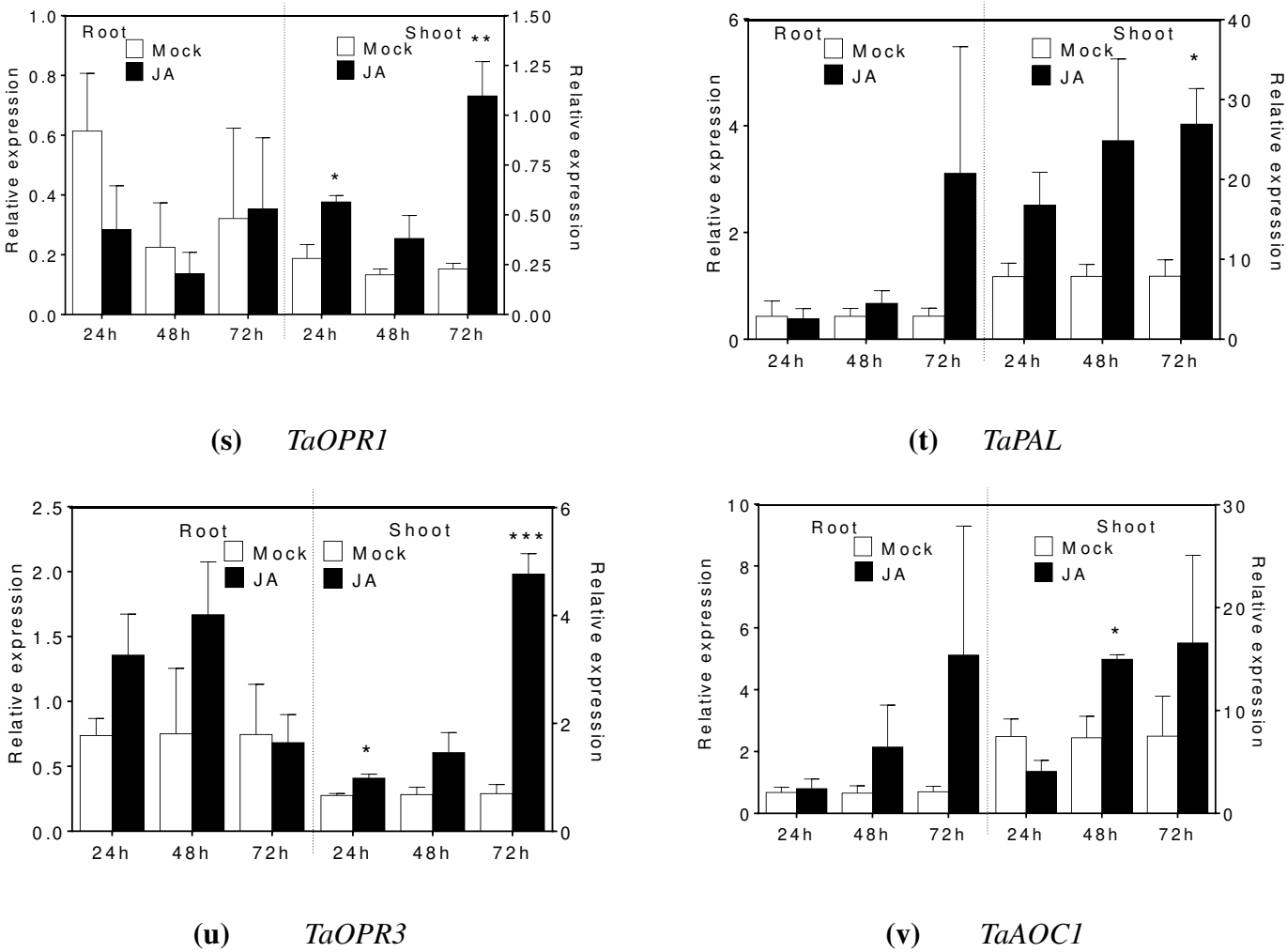
(p) *CHI4*



(q) *TaAOS*



(r) *TaNPR1*



(s) *TaOPR1* **(t)** *TaPAL*

(u) *TaOPR3* **(v)** *TaAOC1*

Fig. S1 Gene expression profiles associated with jasmonate signaling in wheat seedlings (10 day-old). Columns at each time point marked with an asterisk(s) indicate a significant difference between mock- and MeJA-treated samples using two tailed student's t test analysis (P <0.05 *, P <0.01 **, P <0.001***). Data values used in these figures are the same dataset used for generating the heatmap in Figure 2. White and black columns represent mock and MeJA treatments, respectively. Error bars denote the standard error of the mean (n = 3).