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A novel aerobic pink-pigmented facultative, 1-aminocyclopropane-1-carboxylate deaminase producing *Methylobacterium oryzae* sp. nov. isolated from rice

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Abstract
A pink-pigmented, facultatively methylotrophic bacterium, strain CBMB20^T, isolated from stem tissues of rice, was analysed by a polyphasic approach. Strain CBMB20^T utilized 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source and produced ACC deaminase. It was related phylogenetically to members of the genus *Methylobacterium*. 16S rRNA gene sequence analysis indicated that strain CBMB20^T was most closely related to *Methylobacterium fujisawaense*, *Methylobacterium radiotolerans* and *Methylobacterium mesophilicum*; however, DNA–DNA hybridization values were less than 70% with the type strains of these species. The DNA G+C content of strain CBMB20^T was 70.6 mol%. The study presents a detailed phenotypic characterization of strain CBMB20^T that allows its differentiation from other *Methylobacterium* species. In addition, strain CBMB20^T is the only known member of the genus *Methylobacterium* to be described from the phyllosphere of rice. Based on the data presented, strain CBMB20^T represents a novel species in the genus *Methylobacterium*, for which the name *Methylobacterium oryzae* sp. nov. is proposed, with strain CBMB20^T (=DSM 18207^T =LMG 23582^T =KACC 11585^T) as the type strain.

Key Words
Methylobacterium, 1-aminocyclopropane-1-carboxylate, PPFM.

Introduction
The genus *Methylobacterium* includes a group of strictly aerobic, Gram-negative, pink-pigmented facultative methylotrophic (PPFM) bacteria characterized by their ability to utilize single carbon compounds like methanol, formaldehyde via the serine pathway (Green 1992). *Methylobacterium* is classified under the α2 subclass of *Proteobacteria* and presently consists of 22 species with validly published names. Possible mechanisms of plantgrowth promotion by *Methylobacterium* include production of phytohormones, such as indole-3-acetic acid (IAA), cytokinins or vitamins (Basile *et al.* 1985). Here we discuss the formal taxonomic description of a novel species of the genus *Methylobacterium*, Strain CBMB20, isolated from rice tissues with an ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

Methods
Medium used for isolation
Strains CBMB20^T and CBMB110 were isolated from surface-disinfected stem and leaf tissues of rice (*Oryza sativa* L. 'Nam-Pyeoung'). The strains were recovered on ammonium/mineral salts (AMS) medium (Whittenbury *et al*. 1970) supplemented with filter-sterilized cycloheximide (10 mg ml^{-1}) and methanol (0.5% v/v) at 28 °C. The strains were maintained routinely on nutrient agar (NA; Difco) medium, supplemented with 1% (v/v) methanol, or on selective AMS medium.

Scanning electron microscope (SEM)
Scanning electron microscope (SEM) observations were performed on fixed material that was prepared for routine examinations as described by Bozzola and Russell (1998). Samples were critical-point-dried, mounted on stubs, sputter-coated with gold/palladium and visualized by using a Hitachi S-2500C SEM with GEMINI column equipped with a field-emission electron source.

16S rRNA genes
16S rRNA genes were amplified using universal primers: fD1 and rP2 (Weisburg *et al.* 1991) and 16S rDNA sequencing was performed by big-dye primer method using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan).

Nutritional features
Nutritional features were determined using the Biolog Microstation (MicroLog-3, 4.01B). The analysis was

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carried out in Biolog GN2 microtitre plates according to the manufacturer's instructions; the reactions were observed after incubating the plates at 28 °C for 7 days.

Carbon-source utilization tests
Carbon-source utilization tests (excluding biolog) were performed by using a standard protocol described by Green and Bousfield (1982).

Physiological and biochemical characteristics
Other physiological and biochemical characteristics were tested using the API ZYM and API 20NE galleries (bioMe´rieux) following the manufacturer's instructions. Cellular fatty acids were analysed in organisms grown on NA with 1% methanol (v/v) for 48 h.

Cellular fatty acids
The cellular fatty acids were derivatized to methylesters (Sasser 1990) and analyzed by a Gas Chromatograph (Hewlett Packard 6890) using Microbial Identification System (MIDI; Microbial ID) software package.

G+C content
The G+C content of genomic DNA was determined by HPLC analysis using a reverse-phase column (Supelcosil LC-18-S, Supelco) of individual nucleosides, resulting from DNA hydrolysis and dephosphorylation (Mesbah *et al*. 1989).

DNA-DNA hybridization
DNA-DNA hybridization was carried out following the filter hybridization method as described by Seldin and Dubnau (1985).

Results
Strains CBMB20^T and CBMB110 were strictly aerobic, Gram-negative, non-spore forming and forming pink to red-pigmented colonies. Cells were rod shaped, frequently branched and occurred singly or in rosettes on solid AMS and NA medium. Photomicrographs of strain CBMB20 ^{T} are shown in Figure 1.

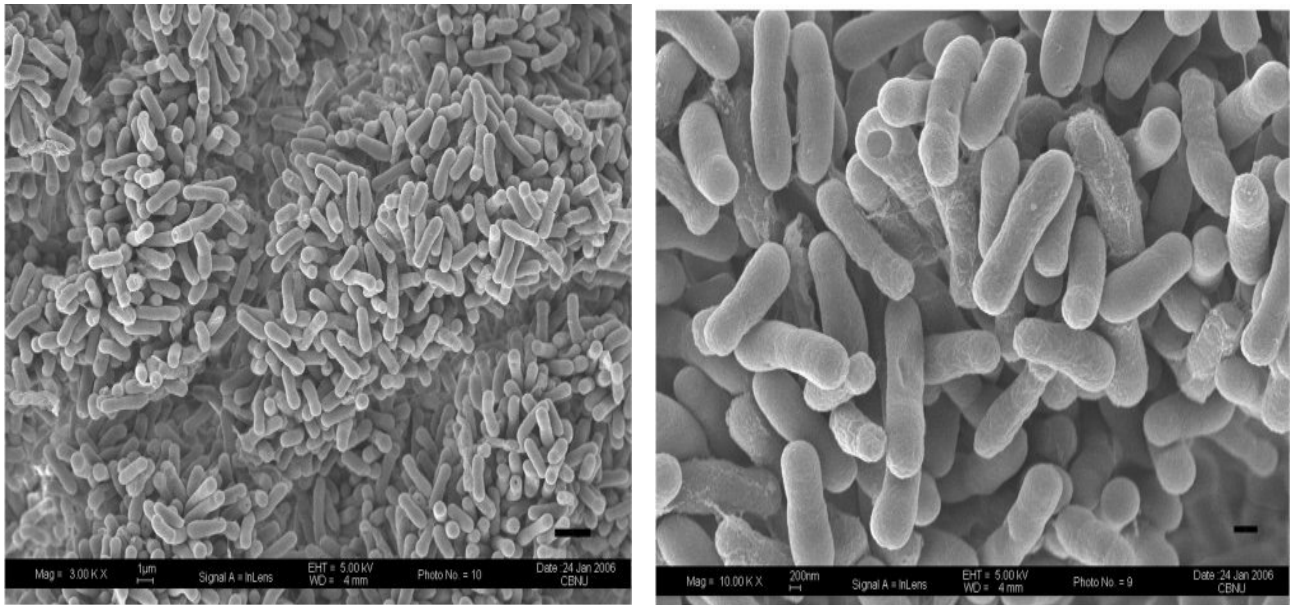

Figure 1. Scanning electron microscope (SEM) photomicrographs of *Methylobacterium oryzae* CBMB20 ^{T} on AMS medium supplemented with 0.5% methanol (v/v) (glutaraldehyde/osmium tetroxide fixation, gold/palladium coating: Hitachi S-2500C). Bar, 1µm and 200nm.

The strain CBMB20 utilized ACC as a nitrogen source and produced ACC deaminase and was phylogenetically related to members of the genus *Methylobacterium*.
The 16S rRNA gene sequence analysis indicated that the strain was most closely related to *Methylobacterium fujisawaense*, *Methylobacterium radiotolerans* and *Methylobacterium mesophilicum* (Figure 2).

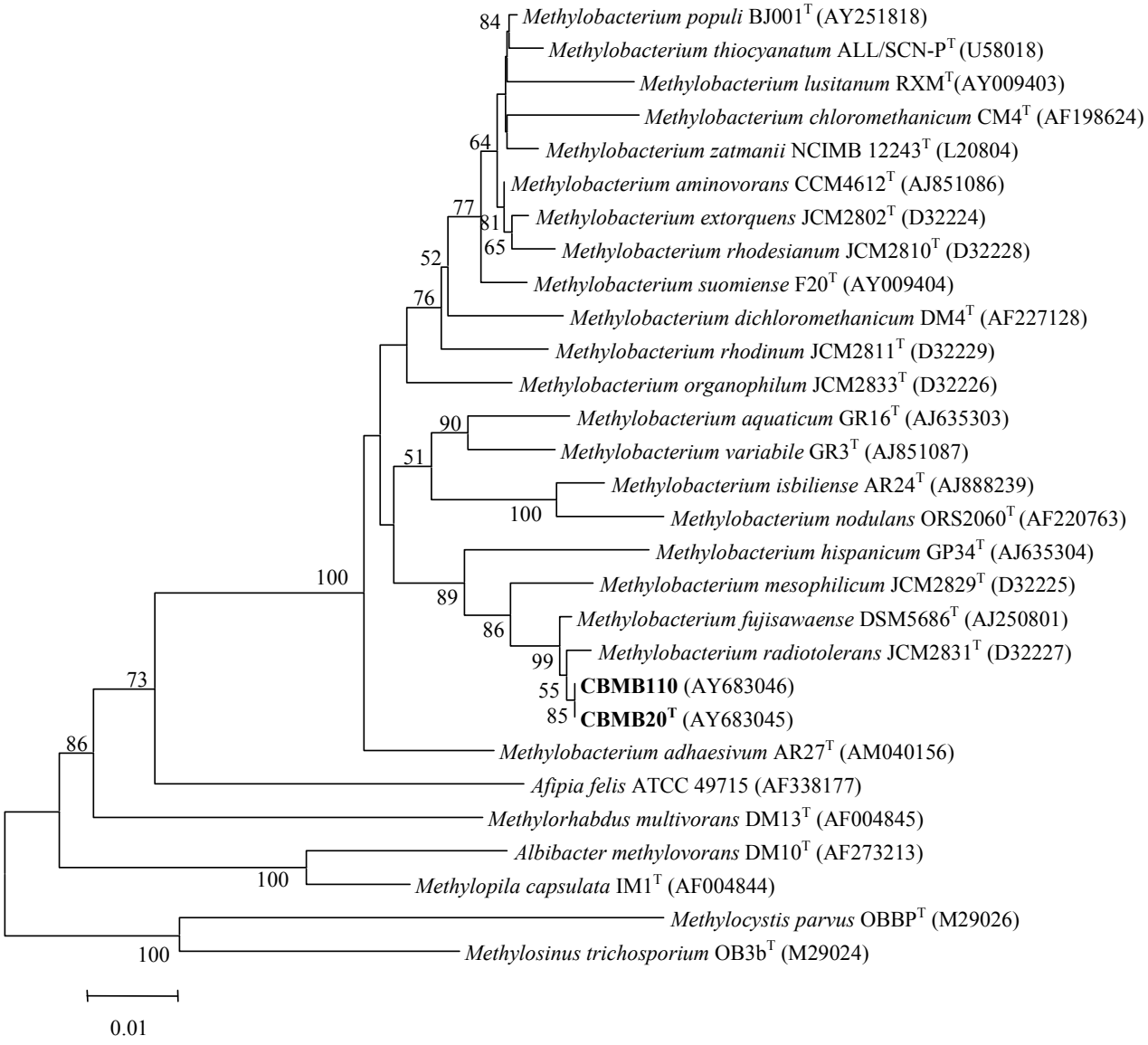

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of the two strains (CBMB20T and CBMB110) and other related species of the genus *Methylobacterium*. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bootstrap values below 50% were not indicated. Bar, 0.01 substitution per site.

However the DNA-DNA hybridization values were less than 70% with the type strains of these species (Table 1).

Table 1. DNA-DNA hybridization values between *Methylobacterium oryzae* CBMB20 T and its closest relatives.

Species	DNA-DNA hybridization (%)
CBMB20T	100.00
CBMB110	88.63
Methylobacterium fujisawaense KACC 10744T	42.09
Methylobacterium mesophilicum DSM 1708T	54.51
Methylobacterium radiotolerans DSM 1819T	63.09

The fatty acid profile of the strains CBMB20T and CBMB110 consisted mainly cis vaccenic acid (C18:1 w7c) and octadecanoate (stearic acid, C18 : 0) (Table 2).

Table 2. Cellular fatty acid compositions (as percentages of the total) of strains CBMB20^T and CBMB110 and related species of the genus *Methylobacterium*.
Summed feature 2 contained one or more of iso-6 $C_{16:1}$ I and/or $C_{14:0}$ 3-OH; Summed feature 3 contained one or more of $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH. 1, CBMB20^T; 2, CBMB110; 3, *M. fujisawaense* KACC10744^T; 4, *M. hispanicum* DSM 16372^T; 5, *M. mesophilicum* DSM 1708^T; 6, *M. organophilum* DSM 760^T; 7, *M. radiotolerans* DSM 1819^T; 8, *M. populi*; 9, *M. suomiense*; 10, *M. lusitanum*; 11, *M. goesingense*. Values are percentages of total fatty acids. Fatty acids representing less than 0.3% in all strains were omitted. ND, Not detected; NR, not reported; ECL, equivalent chain length.

Fatty acids	1	2	3	4	5	6	7	8	9	10	11
$C_{9:0}$	ND	ND	ND	1.2	ND	ND	ND	ND	ND	ND	ND
$C_{12:0}$	ND	ND	ND	4.53	ND	ND	ND	ND	ND	ND	ND
$C_{14:0}$	ND	0.3	ND	2.35	ND	ND	ND	ND	ND	ND	ND
$C_{16:0}$	3.01	3.5	1.96	4.57	3.18	2.98	3.01	6.40	ND	ND	3.3
$C_{18:0}$	4.61	3.66	5.42	4.57	4.1	6.27	5.29	11.9	ND	ND	2.4
$C_{18:0}$ 3-OH	0.77	0.72	0.69	ND	1.52	0.49	0.64	ND	ND	ND	ND
$C_{18:1}$ ω7c	88.2	86.8	88.7	68.5	88.6	88.1	88.8	86.1	84.7	83.1	82.0
Summed Feature 2*	0.77	1.69	0.80	7.97	1.06	0.79	0.81	ND	ND	ND	ND
Summed Feature 3*	1.79	2.1	1.74	6.27	0.84	0.69	0.81	ND	ND	ND	ND
Summed Feature 4*	ND	0.81	ND	ND	ND	ND	ND	ND	ND	ND	ND
Unknown fatty acid 14.959 (ECL)	0.36	0.46	0.47	ND	0.45	0.46	0.50	ND	ND	ND	ND

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained iso-6 $C_{16:1}$ I and/or $C_{14:0}$ 3-OHSummed feature 3 contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:0}$ B**.**

Conclusion

The 16S rRNA sequence similarity data, DNA-DNA hybridization values, and other phenotypic characteristics allowed the strains CBMB20^T and CBMB110 to separate from other members of the genus *Methylobacterium.* Strain CBMB20^T is proposed as the type strain of novel *Methylobacterium* species, for which the name *Methylobacterium oryzae* sp. nov is proposed.

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A novel pink-pigmented facultative *Methylobacterium phyllosphaerae* sp. nov. from phyllosphere of rice

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Abstract
A pink-pigmented, aerobic, facultatively methylotrophic bacterial strain, CBMB27^T, isolated from leaf tissues of rice (*Oryza sativa* L.), was analysed using a polyphasic taxonomic approach. Comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Methylobacterium oryzae, Methylobacterium fujisawaense* and *Methylobacterium mesophilicum*; strain CBMB27 ^{T} showed sequence similarities of 98.3, 98.5 and 97.3 %, respectively, to the type strains of these three species. DNA–DNA hybridization experiments revealed low levels of DNA–DNA relatedness between strain CBMB27^T and its closest relatives. The sequence of the 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) in strain CBMB27 ^{T} differed from those of close relatives. The major fatty acid of the isolate was $C_{18:1}$ ω7c and the G+C content of the genomic DNA was 66.8 mol%. Based on the results of 16S rRNA gene sequence analysis, DNA–DNA hybridization, and physiological and biochemical characterization, which enabled the isolate to be differentiated from all recognized species of the genus *Methylobacterium*, it was concluded that strain CBMB27 ^{T} represents a novel species in the genus *Methylobacterium* for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed (type strain CBMB27^T =LMG 24361 ^{T} =KACC 11716 ^{T} =DSM 19779 ^{T}).

Key Words
ACC, 1-aminocyclopropane-1-carboxylate, PPFM, pinkpigmented, facultatively methylotrophic.

Introduction
Bacteria of the genus *Methylobacterium*, class *Alphaproteobacteria*, consist mainly of a group of pinkpigmented facultatively methylotrophic bacteria with the ability to utilize C1 compounds such as methanol or formaldehyde and other, multicarbon compounds (Green 1992). Cells are strictly aerobic, Gram-negative rods and, at the time of writing, the genus *Methylobacterium* comprised 28 species with validly published names, with *Methylobacterium organophilum* as the type species (Patt *et al.* 1976). Members of the genus *Methylobacterium* are versatile in nature and ubiquitous on plant surfaces, potentially dominating the phyllosphere population (Corpe and Rheem 1989). In this study, a pink-pigmented, aerobic, facultatively methylotrophic bacterial strain CBMB27^T isolated from leaf of rice (*Oryza sativa* L. cv Dong-Jin) was analyzed by a polyphasic taxonomic study.

Methods
Medium used for isolation
Strains CBMB27^T was isolated from surface-disinfected leaf tissues of rice (*Oryza sativa* L. 'Dong-Jin'). The strains were recovered on ammonium/mineral salts (AMS) medium (Whittenbury *et al.* 1970) supplemented with filter-sterilized cycloheximide (10 mg ml^{-1}) and methanol (0.5% v/v) at 28 °C. The strains were maintained routinely on nutrient agar (NA; Difco) medium, supplemented with 1% (v/v) methanol, or on selective AMS medium.

Scanning electron microscope (SEM)
Scanning electron microscope (SEM) observations were performed on fixed material that was prepared for routine examinations as described by Bozzola and Russell (1998). Samples were critical-point-dried, mounted on stubs, sputter-coated with gold/palladium and visualized by using a Hitachi S-2500C SEM with GEMINI column equipped with a field-emission electron source.

16S rRNA genes
16S rRNA genes were amplified using universal primers: fD1 and rP2 (Weisburg *et al.* 1991) and 16S rDNA sequencing was performed by big-dye primer method using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan).

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Nutritional features were determined using the Biolog Microstation (MicroLog-3, 4.01B). The analysis was carried out in Biolog GN2 microtitre plates according to the manufacturer's instructions; the reactions were observed after incubating the plates at 28 °C for 7 days.

Carbon-source utilization tests
Carbon-source utilization tests (excluding biolog) were performed by using a standard protocol described by Green and Bousfield (1982).

Physiological and biochemical characteristics
Other physiological and biochemical characteristics were tested using the API ZYM and API 20NE galleries (bioMe´rieux) following the manufacturer's instructions. Cellular fatty acids were analysed in organisms grown on NA with 1% methanol (v/v) for 48 h.

Cellular fatty acids
The cellular fatty acids were derivatized to methylesters (Sasser 1990) and analyzed by a Gas Chromatograph (Hewlett Packard 6890) using Microbial Identification System (MIDI; Microbial ID) software package.

G+C content
The G+C content of genomic DNA was determined by HPLC analysis using a reverse-phase column (Supelcosil LC-18-S, Supelco) of individual nucleosides, resulting from DNA hydrolysis and dephosphorylation (Mesbah *et al*. 1989).

DNA-DNA hybridization
DNA-DNA hybridization was carried out following the filter hybridization method as described by Seldin and Dubnau (1985).

Results
Cells of strain CBMB27^T were Gram-negative, aerobic, non-endospore forming rods, frequently branched and occurring singly or in rosettes on solid AMS medium and formed pink to red-pigmented colonies. Photomicrograph of strain CBMB27^T grown on solid surface of AMS medium supplemented with 0.5% methanol is shown (Figure 1).

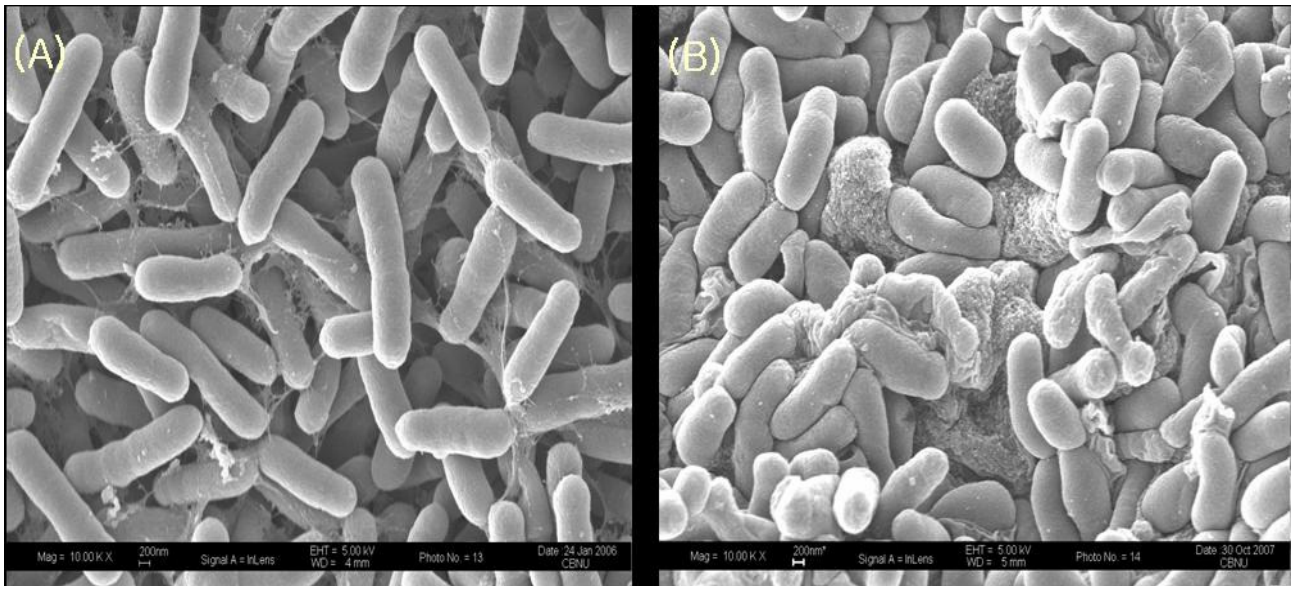

Figure 1. Scanning electron microscope (SEM) photomicrographs of *Methylobacterium phyllosphaerae* CBMB27^T on AMS medium (a) supplemented with 0.5% methanol (v/v) or NA medium (b) supplemented with 1% methanol (glutaraldehyde/osmium tetroxide fixation, gold/palladium coating: Hitachi S-2500C). Bar, 1µm and 200nm.

In this study, a pink-pigmented, aerobic, facultatively methylotrophic bacterial strain CBMB27^T isolated from leaf of rice (*Oryza sativa* L. cv Dong-Jin) was analyzed by a polyphasic taxonomic study. A comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Methylobacterium oryzae*, *Methylobacterium fujisawaense*, and *Methylobacterium mesophilicum,* with which it showed sequence similarity of 98.3, 98.5, and 97.3%, respectively (Figure 2).

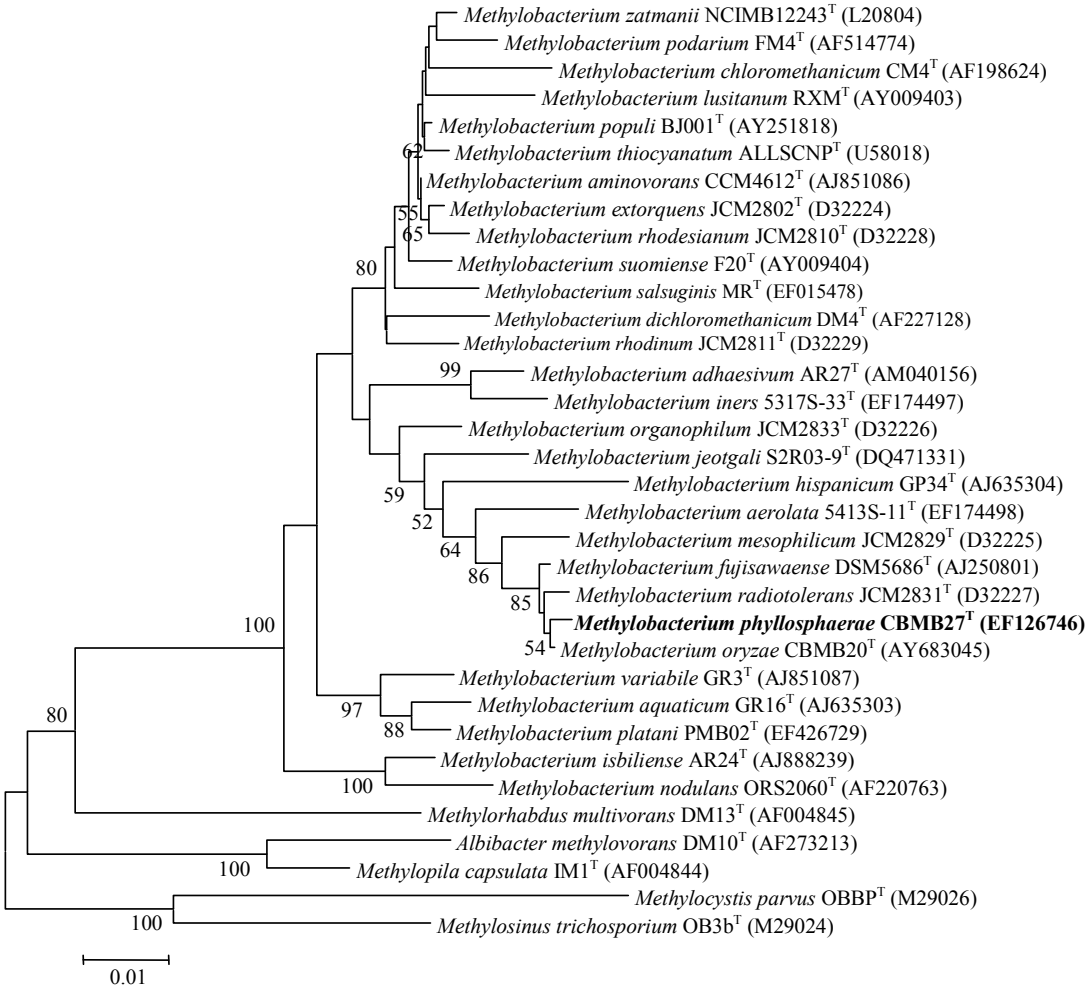

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of the strain CBMB27^T and other related species of the genus *Methylobacterium*. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bootstrap values below 50% were not indicated. Bar, 0.01 substitution per site.

However the DNA-DNA hybridization experiments revealed a low level (< 38%) of DNA-DNA relatedness for the strain CBMB27^T with its closest relatives. The major fatty acid was $C_{18:1}$ ω7c (Table 1) and the G+C content of the genomic DNA was 66.8mol%.

The type strain, CBMB27^T (LMG 24361 ^{T} = KACC 11716 ^{T} = DSM 19779 ^{T}), was isolated from leaf surface of rice (*Oryza sativa* L. cv Dong-Jin). Cellular fatty acids are: *cis*-7-octadecenoate (*cis* vaccenic acid, $C_{18:1}$ ω7c), 75.1%; major hydroxy fatty acids, 11.5%; hexadecanoate (palmitic acid, $C_{16:0}$), 4.1%; octadecanoate (stearic acid, $C_{18:0}$), 4.4% and 3-hydroxy octadecanoate ($C_{18:0}$ 3-OH), 0.9%.

Based on the results of 16S rRNA gene sequence analysis, DNA-DNA hybridization and physiological and biochemical characterization, that differentiated strain CBMB27^T from all recognized species of the genus *Methylobacterium*, it is concluded that strain CBMB27^T represents a new species in the genus *Methylobacterium* for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed (type strain CBMB27 ^{T} = LMG 24361 ^{T} = KACC 11716 ^{T} = DSM 19779 ^{T})**.**

Table 1. Cellular fatty acid compositions (as percentages of the total) of strain CBMB27^T and related species of the genus *Methylobacterium*
Species/strains: 1. CBMB27^T; 2. *M. oryzae* CBMB20 ^{T}; 3. *M. fujisawaense* KACC10744 ^{T}; 4. *M. mesophilicum* DSM 1708 ^{T}; 5. *M. radiotolerans* DSM 1819 ^{T}; 6. *M. extorquens* DSM 1337 ^{T}; 7, *M. adhaesivum* KACC 12195 ^{T}; 8, *M. hispanicum* DSM 16372 ^{T}; 9. *M. organophilum* DSM 760 ^{T}; 10. *M. iners* KACC11765 ^{T}; 11. *M. aerolatum* KACC11766 ^{T}; 12. *M. adhaesivum* KACC12195 ^{T}; 13. *M. platani* KCTC12901 ^{T}.
Values are percentages of total fatty acids; -, not detected. ECL, Equivalent chain-length. Fatty acids representing less than 0.3% in all strains were omitted.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13
$C_{9:0}$	–	–	–	–	–	–	–	1.2	–	–	–	–	–
$C_{12:0}$	–	–	–	–	–	–	–	4.5	–	0.8	–	–	–
$C_{14:0}$	–	–	–	–	–	–	–	2.4	–	0.8	–	–	0.4
$C_{16:0}$	4.1	3.0	2.0	3.2	3.0	2.6	4.5	4.6	3.0	3.3	3.9	2.2	7.6
$C_{17:0}$ ISO 3-OH	11.5	–	–	–	–	9.1	–	–	–	–	–	–	–
$C_{18:0}$	4.4	4.6	5.4	4.1	5.3	2.3	–	4.6	6.3	2.9	2.4	1.1	1.7
$C_{18:0}$ 3-OH	0.9	0.8	0.7	1.5	0.6	–	–	–	0.5	–	1.7	2.0	3.5
$C_{18:1}$ ω7c	75.1	88.2	88.7	88.6	88.8	64.8	79.3	68.5	88.1	82.3	82.6	77.9	81.0
Summed Feature 2*	2.7	0.8	0.8	1.1	0.8	6.3	2.2	8.0	0.8	2.2	3.5	4.3	2.0
Summed Feature 3*	1.1	1.8	1.7	0.8	0.8	12.6	8.5	6.3	0.7	6.5	6.0	11.7	2.2
Summed Feature 4*	–	–	–	–	–	1.31	5.1	–	–	–	–	–	–
Unknown fatty acid 14.959 (ECL)	–	0.4	0.5	0.5	0.5	–	–	–	0.5	–	–	0.9	–

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained iso-6 $C_{16:1}$ I and/or $C_{14:0}$ 3-OH; Summed feature 3 contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH; Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:0}$ B.

Conclusion

The 16S rRNA sequence similarity data, DNA-DNA hybridization values, and other phenotypic characteristics allowed the strain CBMB27^T to separate from other members of the genus *Methylobacterium.* On the basis of these results, strain CBMB27^T is considered to be a novel species of *Methylobacterium*, for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed.

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Ammonia-oxidizing bacteria and archaea in different paddy soils of China

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Abstract
Ammonia oxidation plays an important role in global nitrogen cycle. Little information is available on ammonia oxidation microorganisms in paddy soils. The abundance and composition of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) were investigated by real-time PCR and denaturing gradient gel electrophoresis (DGGE) approaches based on *amoA* genes in selected Chinese soils from Chongqing (CQ, Southwest of China), Honghu (HH, Central China) and Panjin (PJ, Northeast of China) with 10 weeks pot experiments. Little changes in the abundance and community structure of both AOA and AOB were detected among the treatments (without rice, rhizosphere and bulk soil) of the same region paddy soil. The AOB population sizes in CQ paddy soil were lower than those in HH and PJ paddy soils, while the AOA population sizes in CQ and HH paddy soils were lower than those in PJ paddy soil. The *amoA* gene copy numbers of AOA were more than those of AOB in all treatments of the three paddy soils. These results suggested that different region paddy soils determined the population and composition of AOB and AOA.

Key Words
Ammonia oxidizing bacteria and archaea, *amoA* gene, paddy soil, rhizosphere, wetland, nitrogen cycling

Introduction

Ammonia oxidation is the first and rate-limiting step of nitrification and plays a key role in global nitrogen cycle. This process was typically thought to be carried out by ammonia-oxidizing bacteria. Until recently, metagenomic studies demonstrated the potential of ammonia oxidation by mesophilic crenarchaea (Leininger *et al*. 2006; He *et al*. 2007; Prosser and Nicol 2008). Increasing evidences showed that ammonia-oxidizing archaea (AOA) can be detected in wide habitats, such as marine water columns, sediments, estuaries and soils, and may be another major microbial group involved in the ammonia oxidation besides ammonia-oxidizing bacteria (AOB). Extensive researches have paid attention to both AOB and AOA in terrestrial systems, but most of them concern with upland soils, and little information is available in wetland soils. AOB and AOA are aerobes, so it is curious to known their distribution in the flooded anaerobic environment.

Irrigated paddy field is a typical wetland habitat in terrestrial ecosystems. Owing to rice roots releasing O_2, the paddy soil forms a unique environment: the rhizosphere is partially oxic, while the bulk soil is anoxic. The coupling of nitrification and denitrification is favoured by the existence of oxic/anoxic interfaces in paddy soils (Arth *et al*. 1998). As the initial and rate-limiting step of the nitrification and denitrification, ammonia oxidation should even more be studied in paddy fields. China is one of the major rice growers in the world, and has 26% of its total cultivated land growing rice. The suitable environment is provided to study the biogeochemistry and microbiology of the rice paddy. Some studies have shown the abundance and community structure of sulfate reducing prokaryotes, methanotrophs and other important microbial groups (Zheng *et al*. 2008; Liu *et al*. 2009). However, limited information is available on ammonia-oxidizing organisms in paddy soils, especially in different region paddy soils. The aim of this study was to investigate the changes of ammonia-oxidation microbial population size and community structure in different factions (rhizosphere and bulk) of paddy soils collected from different regions of China.

Materials and methods

Soil samples

Paddy soil samples were collected from three different regions of China: CQ, purple paddy soil with wheat-rice rotation from Chongqing city (N29.83°, E106.43°), southwest of China; HH, alluvial paddy soil with rapeseed-rice rotation from Honghu city (N29.48°, E113.27°), Hubei Province, central China; PJ, coastal saline paddy soil with single-season rice from Panjin city (N41.12°, E122.06°), Liaoning Province, northeast of China. Some soil characteristics were listed in Table 1. All soil samples were air dried and sieved through a 2 mm mesh and stored at room temperature for subsequent pot experiments.

Table 1. Selected properties of the original soil samples

Region	pH (H_2O)	organic matter ($g\ kg^{-1}$)	alkali-hydrolysable N ($mg\ kg^{-1}$)	available phosphorus ($mg\ kg^{-1}$)	available potassium ($mg\ kg^{-1}$)
CQ	8.2	26.4	173	8.20	224.82
HH	7.6	23.0	171	1.17	220.52
PJ	8.1	13.6	77.0	5.92	221.97

Pot experiment

PVC pots (18 cm diameter, 20 cm height) were used to load the soil samples. Each soil was set up two treatments with triplicate: one treatment was rice planted and the other no rice planted. The treatment growing the rice was divided into bulk soil and rhizosphere with a root bag (37 μm nylon mesh, 5 cm diameter, 10 cm height, and one plant per bag). Each pot was filled with 3 kg of soil, including 0.2 kg soil in the root bag. The pots were maintained waterlogged until harvest. Rice seeds were pretreated and germinated. After three weeks by water culture, uniform seedlings were transplanted into the pots. Then, the rice plants were cultivated for 10 weeks in a greenhouse.

Sample collection

At the tenth week of the pot incubation, rice plants were harvested and meanwhile the rhizosphere and bulk soils were sampled from each treatment. All the samples were divided into two parts: one was stored at 4°C for chemical analysis and the other frozen at -80°C for subsequent DNA extraction and molecular analysis. The soil chemical properties at the end of the pot experiments are listed in Table 2.

Table 2. Selected chemical properties of the paddy soils at the end of the pot experiment

Region	Treatment*	pH	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)
CQ	n-CQ	7.4±0.1 c	370±6 f	0.91±0.01 cd
	r-CQ	7.0±0.0 a	189±27 c	0.91±0.02 cd
	b-CQ	7.3±0.0 b	323±21 e	0.72±0.05 bc
HH	n-HH	7.4±0.0 c	253±12 d	0.87±0.01 cd
	r-HH	7.0±0.1 a	110±16 b	0.88±0.07 cd
	b-HH	7.2±0.1 b	216±23 c	0.82±0.07 cd
PJ	n-PJ	7.7±0.1 d	268±22 d	1.00±0.05 d
	r-PJ	7.0±0.1 a	45.1±13.2 a	0.65±0.16 b
	b-PJ	7.5±0.1 c	187±16 c	0.50±0.12 a

***** Treatment: without the rice with n, rhizosphere with r and bulk soil with b. Values are mean±SD (n = 3). Values within the same column followed by the same letter are not significantly different at $P < 0.05$.

Molecular analyses

DNA was extracted from 0.5 g frozen paddy soil samples using UltraClean™ Soil DNA Isolation Kit following the manufacturer's protocol. The real-time PCR quantification analysis of *amoA* genes was conducted on an iCycler iQ5 Thermocycler, and the PCR-DGGE analysis of *amoA* fragments was performed as described before (He *et al*. 2007; Shen *et al*. 2008).

Results

Abundance of AOB and AOA in paddy soils

The *amoA* gene copy numbers of AOB and AOA were assessed using quantitative PCR. The bacterial *amoA* gene copy numbers in soils ranged from 1.41×10^4 to 2.87×10^5 copies per gram dry soil, while the archaeal *amoA* gene copy numbers ranged from 3.95×10^6 to 2.87×10^7 copies per gram dry soil (Figure 1). No significant differences in the abundance of AOB and AOA were detected among the treatments (without rice, rhizosphere and bulk soil) in each paddy soil (Figure 1.). Within the AOB abundance, the AOB population size in CQ paddy soil was remarkably lower than that in HH and PJ paddy soil. While for the AOA abundance, PJ paddy soil was significantly higher than CQ and HH paddy soils. Ratios of AOA to AOB *amoA* copy numbers were ranged from 22.9 to 667 in all treatments, indicating that the AOA population size among the treatments in three region paddy soils was higher than AOB.

DGGE profiles of AOB and AOA communities in different region paddy soils

The DGGE patterns of AOB indicated clear variations among different region paddy soils. However, the band patterns of AOB were similar in the same region paddy soil, regardless of without rice or rhizosphere or bulk soil. The correspondence analysis (CA) ordination diagram of AOB communities is shown in Figure 2,

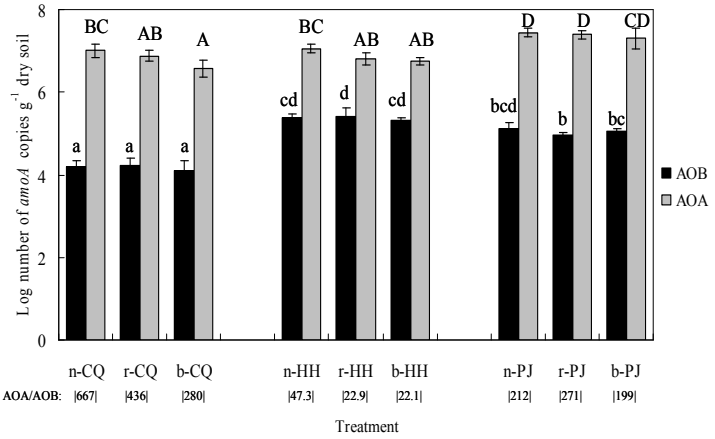

Figure 1. Abundances of AOB and AOA in different region paddy soils (CQ, HH and PJ) with the treatments of without rice (with n), rhizosphere (with r) and bulk (with b). Values are mean±SD (n = 3). Within AOB or AOA *amoA* copy numbers, values followed by the same letter are not significantly different at P < 0.05. Ratios of AOA to AOB *amoA* copy numbers are shown at the bottom with each treatment.

and individual points on the two-dimensional biplot represented AOB communities with different treatment in the three paddy soils. Sixty three percent of the variance in community structure was explained by four eigenvectors. The first and second eigenvectors, plotted on the x and y axes, explained 32% and 18% of the variation, respectively; thus, 50% of the cumulative variation was explained. The difference between the communities with respect to the first and second eigenvectors was the distance between the points representing the communities on the ordination diagram. A comparison of the distances showed that the AOB communities from the same paddy soil had the most similar structure. Take CQ paddy soil as example, the AOB communities associated with the treatments of without rice, rhizosphere and bulk were separated less than 0.5 standard deviation. However, the AOB communities from the different paddy soils were the most dissimilar and were separated by approximately 1 or more than 1 standard deviation. The CA supported visual observation of the DGGE gel, indicating that the different treatments (without rice, rhizosphere and bulk soil) did not influence the species data while the different region paddy soils did affect community structure.

The DGGE results of AOA also revealed that band patterns had little change among the treatments of without rice, rhizosphere and bulk soil in the same region paddy soil. There were also obvious differences among the three region paddy soils in DGGE patterns. From the CA ordination diagram of AOA communities, the treatments from the same paddy soil were clustered together and three distinct clusters were observed according to the three region paddy soils (Figure3). Eighty seven percent of the variance in community structure was explained by four eigenvectors, thus the majority of the changes in community structure can be attributed to the different region paddy soils.

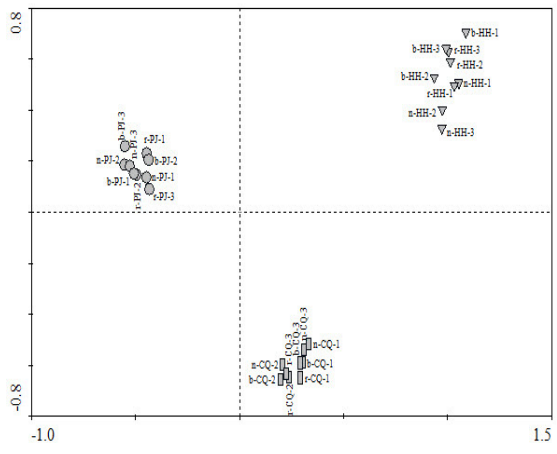

Figure 2. Correspondence analysis (CA) ordination diagram of AOB communities with the treatments of without rice (with n), rhizosphere (with r) and bulk (with b) in different region paddy soils (CQ, HH and PJ) generated by the DGGE profiles. Eigenvectors for first and second ordination axes (x and y axes) explain 32% and 18% of the variation, respectively.

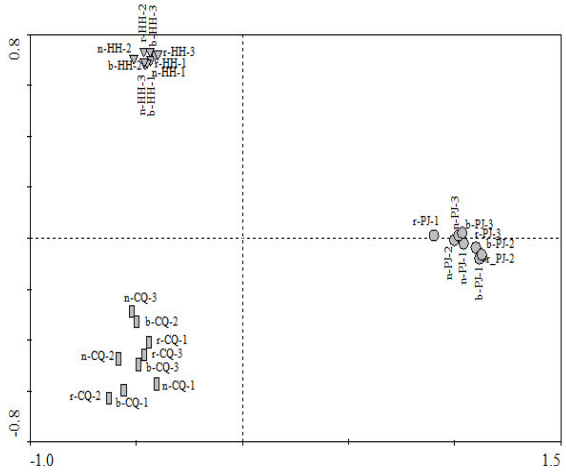

Figure 3. Correspondence analysis (CA) ordination diagram of AOA communities with the treatments of without rice (with n), rhizosphere (with r) and bulk (with b) in different region paddy soils (CQ, HH and PJ) generated by the DGGE profiles. Eigenvectors for first and second ordination axes (x and y axes) explain 51% and 23% of the variation, respectively.

Conclusion

This study found that both AOB and AOA abundance and community composition existed few changes among the treatments (without rice, rhizosphere and bulk soil) in each paddy soil, but distinct differences were observed among the three paddy soils, suggesting different region paddy soils affected the population and composition of AOB and AOA. We also highlighted the need to further study the AOB and AOA growth and activity in paddy soils to assess their contribution in N cycle in paddy soils, together with denitrification and annamox processes.

Acknowledgements

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Assessing Tolerance of Rhizobial Lentil Symbiosis Isolates to Salinity and Drought in Dry Land Farming Condition

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Abstract
Due to nutrient including nitrogen deficiency, vegetable production is restricted in most of semi arid and especially saline lands, and nitrogenous chemical fertilizers in these lands cause the salinity to increase, chemical fertilizer consumption may be economically expensive. So, nitrogen fixation biofertilizer application would be important. In this research 220 rhizobial bacteria were isolated from two lentil dry land farming flat plains of Moghan and Koohin. BTB +YMA culture media with different levels of salinity by NaCl was used in a saline tolerance test of rhizobial lentil symbiont strains. Among 184 rhizobial strains, 101 strains were completely sensitive (EC=10 ds/m) and 10 strains were superior strains which were completely tolerant to salinity. Altogether Koohin flat plain rhizobacteria were more saline tolerant than Moghan flat plain rhizobacteria. Drought tolerance test of rhizobial strains was performed in PEG 6000 +YMA culture media. The drought level tolerance was evaluated based on optical density of bacterial suspension in this test. Rhizobacteria were grouped in 4 levels based on their drought tolerance as completely tolerant, tolerant, sensitive and completely sensitive using their optical density. Among 34 super strains which are completely tolerant, 9 super strains of Moghan flat plain and 25 super strains of Koohin flat plain were selected. At last we hope that the super strains of this research can be used in subsequent studies.

Key Words
Salinity, drought, NaCl, PEG, *Rhizobium*.

Introduction
About one-third of the land area of the world comprises arid and semi arid climates and approximately 15% of these land areas are affected by saline. However salinity and drought would suppress rhizobia growth and multiplication but there are some strains which can withstand high levels of drought and salinity and survive. Soil salinity is caused by the accumulation of soluble salts of calcium, magnesium and sodium, mostly as chlorides and sulphates (Singleton *et al*. 1994). About 400-950 million hectare of the world agricultural lands are affected by salinity (Allen *et al*. 1995; Bernstein *et al.* 1983). The whole saline land area of Iran is more than 15 million hectare which comprises 10% of the total area of the country and the salinity is increasing due to the excessive irrigation, saline water utilization and incorrect consumption of chemical fertilizer (Allen *et al*. 1995). Plant production is limited in these arid and semi arid land area because of nitrogen deficit (Worrall and Roughly 1976) and Nitrogen consumption may increase plants tolerance to salinity, however nitrogenous chemical fertilizers consumption would cause more salinity of these land area and it wouldn't be cost effective. So it is important to consider about Biological Nitrogen Fixation and Biological fertilizers utilization especially microbial fertilizers (Cordovilla 1996; Pessaraki and Tucker 2005).

Material and Methods
Sampling and Rhizobial isolates preparation
To investigate drought and salinity tolerance of rhizobial isolates symbioant of lentil, lentil nodule samples were collected from different agricultural field sites in Moghan and Koohin flat plains in Ardebil and Zanjan provinces, respectively. After washing the root system of the lentil plants, a well formed, healthy pinkish nodule on the tap root of each sample was carefully cut out with a portion of the root attached to the nodule. Then the nodules were transferred to the laboratory for further experiments in small desiccators containing silicagel and were coded individually. The samples related to Moghan flat plain were coded 1-110 and 110-220 for Koohin. To isolate rhizobial strains, the nodules were sown in water and they were surface sterilized for 5 minutes in 0.1% mercuric chloride in water and repeatedly washed with sterile water to get rid of the chemical. The nodules then washed in 70% ethyl alcohol for 3 minutes followed by more washing with sterile water. Then they were crushed with a sterile glass rod in a small aliquot of sterile water. A small aliquot of the suspension was transferred on Congo red incorporated yeast extract mannitol agar medium to obtain sparse and distinct colonies (Subba Rao 1999). At the end of incubation period, macroscopic and

microscopic observations were performed for grown colonies. Gram staining and plant infection test (PIT) were carried out to confirm rhizobia colonies. The purified isolates were transferred to agar slants in 2-5 °c and also preserved in conservation media of YM broth containing glycerol in -80°c (Vincent 1982). 220 rhizobial isolates were totally recovered from the nodules of the collected lentil plant roots.

Evaluation of the isolates tolerance to salinity
The ability of the rhizobial isolates to withstand salinity was evaluated by their growing at different concentrations of NaCl salt with different electrical conductivities (EC) of 10 to 50 ds/m in bromothymol blue (BTB) incorporated yeast extract mannitol agar medium. Rhizobial strains were inoculated and after incubating, well-grown bacteria which have been changed the colour of medium from blue to yellow were supposed as tolerant isolates.

Evaluation of the isolates tolerance to drought
The drought tolerance of the isolates was also examined, using Poly Ethylene Glycol 6000 (PEG 6000) in YMB medium with Different concentrations (Burlyn E. Michel & merrill R. Kaufmann 1973) using the following formula:

$$Water\ potential\,(wp) = -(1018e-2)c-(1.18e-4)c^8 2+(2.67e-4)ct+(8.39e-7)c^8 2T$$

After inoculating the isolates in sterile condition and incubating on a rotator shaker for 72 hrs in 28°c, their optical density in 570 nm were measured. In fact optical densities were decreased by increasing of PEG amount in the media. The results were analysed in Excel software.

Results and discussion
The number of lentil samples collected from Moghan flat plain in Ardebil province and Koohin flat plain in Zanjan province were equal and total samples number was 220. 214 bacterial strains were isolated on bromothymol blue incorporated yeast mannitol agar media. Isolated colonies of YMA+ Congo red were gram negative, white, translucent, glistening, elevated 3-5mm colonies with entire margins. These colonies changed the colour of media containing BTB to yellow colour. During subculturing, bacterial isolates were purified and refrigerated in 2-5 °c. Plant infection test (PIT) was carried out in tubes with a proper media for lentil legumes in the growth chamber. It was demonstrated that among 214 isolates, 25 isolates were not *rhizobium legominosarum* bv. *Viciae* (lenti)(*RLV*). The results of salinity tolerance experiment illustrated that among 189 rhizobial strains symbioant with lentil plant (*RLV*) , the number of grown strains on different levels of salinity was as followed: 1) EC=10 ds/m, 101 strains(53.44%) which was defined as completely sensitive, 2) EC= 20 ds/m, 93 strains (49.21%), sensitive, 3) EC= 30ds/m, 71 strains (37.57%) defined as partially tolerant, 4) EC = 40ds/m, 35 strains (18.52%) tolerant and 5) EC=50ds/m, 35 strains, completely tolerant (Table 1) .Although 25 grown strains in EC=50 ds/m changed the color of their BTB+YMA media from blue to yellow but the colonies didn't grow strongly and typically, while 10 rhizobial strains grew completely were supposed as superior strains. Among 110 isolated Moghan flat plain strains, 46 strains (41.82%) grew on EC= 10 ds/m while the number of grown strains in similar salinity for Koohin flat plain was 55 strains (50%).

It can be resulted that the collected samples of Koohin flat plain had much more salinity tolerant strains than Moghan. However extreme condition suppresses the multiplication and growth of rhizobial strains but there are some strains which can tolerate high amounts of salt and survive (Lakshmi and Subbarao 1984; Vmanchanda and Garg 2008).

Subba rao (1994) had observed a significant difference in the growth of different inoculated rhizobial strains on a salinized YMA medium and reported that rhizobial tolerance to salinity is more than their host plants. Subba rao *et al*. (1999) reported that Rhizobial strains which were able to make an efficient symbiosis in high levels of salinity may not be necessarily originated from saline soils. In return Duca *et.al*. reported that the collected isolates from dry and saline soils are more successful to make root nodules in saline conditions. The researches have been proved that some free-living bacteria (Saprophytes) are able to survive in drought or saline stress conditions.

Table 1. The number of grown rhizobial strains in different levels of salinity.

Grown rhizobial strains		Different levels of salinity	Tolerance to salinity
Percentage	Number	EC(ds/m)	
53.44	101	10	Completely sensitive
49.21	93	20	sensitive
37.57	71	30	Partially tolerant
18.52	35	40	tolerant
18.52	35	50	Completely tolerant
5.29	10	50	Superior strains

The results from assessing the tolerance of symbioant rhizobacteria showed that among 34 superior tolerant isolates of salinity experiment, 6 strains (17.65%) were completely sensitive to drought stress and 8 strains were sensitive and 15 strains were grouped as drought tolerant and finally 5 strains were defined as completely drought tolerant(Table 2). Drought stress is one of the major environmental factors affecting most crops and decreasing crop yield (Vmanchanda and Garg 2008). The population of soil bacteria decreases along the moisture stress however it would not be lost completely and certain soil bacteria can resist these extreme dry conditions. These microorganisms utilize the water preserved in the micro porous of soil and survive by their minimum metabolic activities.

Table 2. The tolerance of lentil symbioant rhizobial strains to drought stress.

Number of strains	Tolerance to drought	Optical density (OD)
6	Completely sensitive	OD<0.3
8	sensitive	OD=0.3-0.4
15	tolerant	OD=0.4-0.5
5	Completely tolerant	OD>0.5

Conclusion

The results of the experiments demonstrated that we can isolate and purify drought and salinity completely tolerant rhizobial isolates from Iranian dry land farming soils.
Although the strains isolated from Moghan flat plain showed fewer tolerance to salinity and were placed in completely sensitive (46 isolates) and sensitive (42 isolates) groups and they could not tolerate drought condition and the number of all groups from completely sensitive to completely tolerant were less than Koohin flat plain isolates. Hence, it can be concluded that drought tolerance is parallel with salinity tolerance for rhizobia strains. Zahran (1999) also reported that salinity sensitive bacterial strains can not resist against high levels of drought and it is also related to salinity. We hope more supplementary investigations would be conducted to introduce the superior strains to produce industrial and commercial biological fertilizer symbioant lentil.

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Bacillus-like phosphobacteria in agronomic volcanic soils from Chile

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Abstract
Here we evaluated the occurrence of *Bacillus*-like phosphobacteria (BLP) in rhizospheric soils from diverse grasslands established on agronomic volcanic soils of Southern Chile. BLP were grown on culture media containing insoluble phytate and phosphate as sole phosphorus (P) source. Additionally, phytase gene was detected by PCR using a degenerate primer set for *Bacillus* phytases. Sequencing of DNA fragments amplified by PCR, revealed the presence of *Paenibacillus* carrying phytase gene close to 3-phytase. This study reveals that Chilean volcanic soils are a reservoir of BLP populations which can present mechanisms involved in the expression of phytases. To our knowledge, this is first report on detection and characterization of *Bacillus* phytase gene in rhizospheric soils.

Key Words
Bacillus. phytase, phytate, rhizosphere, phosphobacteria, volcanic soils

Introduction
The biofertilizers are live microorganisms that are able to colonize the rhizosphere or internal tissues of plants, promoting the growth of host plant through increase supply or availability of nutrients, such as nitrogen and P (Richardson *et al.* 2009). Bacteria with capacity to release phosphate from insoluble P forms by organic acid production and enzymatic activity (i.e. phytase), also known as phosphobacteria, are currently studied and commercialized to improve the growth, yield, and quality of crops as well as for biotechnological applications in other fields, such as animal and human nutrition, aquaculture and environmental protection (Jorquera *et al.* 2008). In this context, *Bacillus* is one of most isolated and studied phosphobacterial groups and commonly is suggested as potential biofertilizers. However, the occurrence, genomics and dynamics of BLP in agronomic soils is clearly unknown and poorly studied. The objectives of the present study were i) to evaluate the occurrence of BLP in rhizospheric soils from diverse grasslands established on agronomic volcanic soils of Southern Chile, and ii) to evaluate the presence of genetic traits involved in the expression of phytase which is a enzyme capable to release phosphates from phytate in soils.

Methods
Sampling
Rhizospheric soil samples were taken from three grasslands located in the La Araucania and Los Ríos regions from Southern Chile, and inmediatelly transported to laboratory for their analysis. The chemical properties of rhizospheric soils are shown in the Table 1.

BLP isolation
Bacillus-like were isolated according to standard protocol (Holt and Gerhardt 1994). Rhizospheric soil samples were treated at 80°C for 10 min and aliquotes were plated on phytase-screening medium (Kerovou *et al.* 1998) to detect *Bacillus*-like isolates with phytate-mineralization capacity.

Phytase gene detection by PCR
Based on current literature and public database, a degenerate primer set for *Bacillus* phytases described by Tye *et al*. (2002) was chosen and used to detect phytase gene in rhizospheric isolates of BLP (Figure 1, B). The isolates showing positive phytase signal were selected and characterized by 16S rRNA gene sequencing. The presence of phytase gene was also confirmed by sequencing. The DNA sequences obtained in this study were compared with those present in Genbank database by BLAST tools.

Results and Discussion
Despite of significant differences in the chemical properties among the soils sampled (except % organic matter), the results showed that between 77-91% of cultured *Bacillus*-like showed capacity to solubilise phosphate and/or mineralize phytate on agar (Figure 1, A). Two grasslands showed high percentages of

Bacillus-like isolates with phytate mineralization capacity, 66 and 75% for grassland no.1 and no.2, respectively. In contrast, the higher percentage in grassland no.3 was observed in *Bacillus*-like isolates with both phytate mineralization and phosphate solubilization activity. *Bacillus*-like isolates with capacity to utilize insoluble P forms (phosphates and phytates) have commonly isolated from rhizosphere of different plants and suggested as plant-growth promoting rhizobacteria (Idriss *et al.* 2002; Richardson *et al.* 2009). The analysis of DNA sequences revealed the presence of *Paenibacillus* carrying phytase gene close to 3-phytase reported in *Bacillus* strains (Figure 1, C). The great biotechnological potential of *Paenibacillus* in different industrial processes and in sustainable agriculture has been recently revised (Lal and Tabacchioni 2009). On other hands, some studies have failed in detection of bacterial phytase in environmental isolates, especially *Bacillus* (Hill *et al.* 2007). The specific detection of phytase gene will allow further studies focused on the dynamics and expression of phytase gene in the rhizosphere plants, with the consequent importance in the application of *Bacillus*-based biofertilizers to improve the P nutrition in plants. To our knowledge, this is first report on detection and characterization of *Bacillus* phytase gene in rhizospheric soils.

Table 1. Average values of selected chemical properties of rhizospheric soils.

	Grassland		
	1	2	3
pH H_2O	5.0-5.1	5.3-5.8	5,7-5,9
P (ppm)	5	36	8
K (ppm)	70	454	224
Organic matter (%)	20	19	19
Al Saturation (%)*	30.8	2,8	0,5

*Calculated as = Al/cation exchange capacity [∑(K, Ca, Mg, Na and Al)]×100

Tabla 2. Average values of *Bacillus*-like isolated from diverse grassland.

Grassland	SP (%)	MP (%)	SPMP (%)	NSM (%)	TOTAL (%)	TOTAL BLP (%)
1	1.4	65.9	11.3	21.4	100	77.2
2	5.2	74.7	15.8	4.3	100	90.5
3	2.5	17.5	66.0	14.0	100	83.5

SP=*Bacillus*-like that solubilize phosphate; MP= *Bacillus*-like that mineralize phytate; SPMP= *Bacillus*-like that solubilize phosphate and mineralize phytate; NSM= *Bacillus*-like that no solubilize phosphate and/or mineralize phytate.

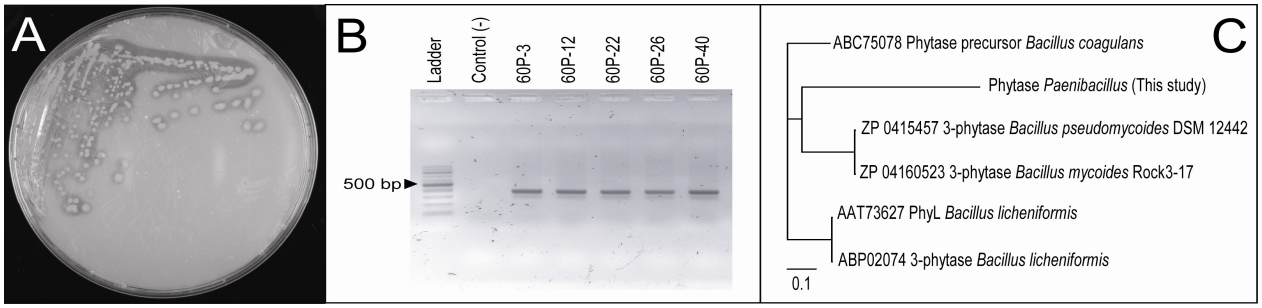

Figure 1. (A) Phytate-mineralization on agar by BLP. (B) PCR amplification of phytase gene in environmental isolates of BLP. (C) Neighbor-Joining tree showing phylogenetic affiliation of detected phytase and the closest relative phytases in Genbank database.

Conclusions

This study shows the high occurrence of *Bacillus*-like phosphobacteria in rhizospheric soils from diverse grasslands established on agronomic volcanic soils of Southern Chile. Also, this study gives evidence for the presence in these soils of *Paenibacillus* spp. carrying mechanisms involved in the expression of phytases.

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Biocontrol of soil-borne Fusarium wilts of tomato and cabbage with a root-colonizing fungus, *Penicillium* sp. EU0013

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Abstract
The soil-inhabiting fungal pathogen *Fusarium oxysporum* can result of severe losses in many plants. We investigated the biocontrol effect of a novel root-colonizing fungus, *Penicillium* sp. EU0013, to Fusarium wilt diseases of tomato and cabbage. Application of EU0013 at inoculum concentration of 10^6 conidia /g to the potting mix resulted in 78 and 74% reduction of the diseases in tomato and cabbage, respectively. With the increase of applied EU0013 concentration, disease severity was reduced. Recovery of EU0013 from tomato and cabbage roots was 39 to 81% and 36 to 79%, respectively.

Key Words
Biocontrol, soil-borne wilt diseases, root colonization, plant growth promoting fungi.

Introduction

Soil-borne wilt diseases caused by *Fusarium* spp. are difficult to control. Among the different farm management practices, crop rotation is known to be less effective in controlling this pathogen because of its wide host range. Broad-spectrum biocides, such as methyl bromide have been widely used to manage soil-borne diseases caused by *Fusarium* spp., however such soil fumigants have hazardous effects on the environment (Blancard 1993). Similarly breeding cultivars that are resistant against soil-borne diseases is difficult due to mutation in *Fusarium* spp; therefore research has been focused on the biological control (Fravel *et al*. 2003).

A novel plant growth promoting fungus (EU0013) was recently isolated from eucalyptus roots (Teshima and Sakamoto 2006). From morphological features of the conidiophores and sequence data on the ITS region of rDNA, the EU0013 was identified as *Penicillium* sp. EU0013 colonized cabbage roots and significantly increased seed germination and seedling growth. We investigated EU0013 as a candidate for biocontrol agent to Fusarium wilt of tomato and cabbage in controlled environmental conditions.

Materials and Methods

In vitro antagonism of EU0013
Using dual culture technique, the EU0013 and causal agents of Fusarium wilt of tomato, *Fusarium oxysporum* f.sp. *lycopersici* CU1 and cabbage, *F. oxysporum*. f.sp. *conglutinans* K124F were grown in the same Petri dish by inoculating fungal culture distantly apart from each other on PDA media, and then incubated at 25 ^{o}C in dark for 7 days. The inhibition of the pathogens were recorded either in formation of inhibition zone or overgrowth to the pathogen by the EU0013. The colony diameter of pathogens in dual culture was measured and growth inhibition was expressed as percentage of the control (Baker and Cook 1974).

Effect of EU0013 on the development of Fusarium wilt in tomato and cabbage
Biocontrol effect of *Penicillium* sp. EU0013 against tomato and cabbage wilt was studied in growth chamber conditions. Seeds of tomato cv. Oogatahukuju were grown in 5x5x5 cm plastic pots containing autoclaved potting mix (peat and vermiculite mixed 1:1 v/v) placed in a lighted growth chamber at 25^{o}C during day (16h) and 18 ^{o}C at night (8h). Different concentrations (10^2, 10^3, 10^4, 10^5, and 10^6 conidia /g of potting mix) of EU0013 were applied 1 week after sowing and 10^5 conidia /g of CU1 was applied at 2 weeks after sowing the seeds. After 10 days of inoculation, seedlings were monitored regularly for external symptoms of Fusarium wilt for a period of 40 days. Disease severity was evaluated by the percentage of leaves with symptoms (yellowing and wilting) to the total number of leaves.

Root colonization by EU0013 was examined by sampling small root sections. Each root segment was surface sterilized by immersion in 70% ethanol, followed by rinsing in sterile water and incubation at 25 ^{o}C in dark for 1 week. Root colonization was scored positive, when a typical EU0013 colony developed. Root staining method reported by Giovanetti and Mosse (1980) was used for hyphal observation of EU0013 in tomato root

tissues under light microscope. Effect of EU0013 on the development of cabbage wilt was studied using same protocol as described above. Cabbage cultivar Shikidori was used in this study.

Results and Discussion

Efficacy of Penicillium sp. EU0013 on the growth of Fusarium wilts pathogens.

In dual culture experiment, EU0013 formed an inhibition layer near CU1 and K124F without any physical contacts. EU0013 resulted in 30.6% and 29.6% reduction radial of colony growth in CU1 and K124F, respectively, compared to the control (Table 1). This inhibition zone formation indicates that EU0013 produces some antifungal compounds which inhibit the growth of these pathogens. The nature and composition of such compounds is not yet known.

Table 1 Effect of EU0013 on the growth of *Fusarium oxysporum* f.sp. *lycopersici* CU1 and *Fusarium oxysporum*. f.sp. *conglutinans* K124F on Potato Dextrose Agar (PDA) after 10 days of incubation.

EU0013	Growth of CU1		Inhibition zone (mm)	Growth of K124F		Inhibition zone (mm)
	Colony diameter (mm)	inhibition[A] (%)		Colony diameter (mm)	inhibition[A] (%)	
EU0013 (+)	55.5± 0.43	30.6±0.95	3.5 ± 0.05	53.42±0.34	29.56 ±0.59	3.44 ±0.07
EU0013 (-)	80.3± 0.76	-	-	75.84 ±0.21	-	-

Data represent mean±SE (n=5); (+) with EU0013; (-) without EU0013

[A]Inhibition (%) = (1-colony diameter of CU1 or K124F in EU0013 (+)/ colony diameter of CU1 or K124F EU0013 (-)) x100

Efficacy of Penicillium sp. EU0013 on the disease development of tomato and cabbage wilt

Disease severity and percent disease reduction varied significantly (P <0.05) with the concentration of EU0013 (Table 2). In the absence of EU0013, both CU1 and K124F exhibited severe disease (Figure 1). However, prior application of EU0013 significantly reduced the disease severity in tomato and cabbage. The highest disease reduction (78%) in tomato plants was obtained by prior application of EU0013 at 10^6 conidia/g of potting mix, followed by application at 10^5 conidia/g. Disease reduction at these two levels was not significantly different from each other. Application of the same levels of conidial concentration of EU0013 to cabbage resulted in 74 and 70% disease reduction, respectively.

EU0013 recovery from roots of tomato and cabbage varied 39 to 81% and 36 to 79%, respectively (Table 3). The root colonization by EU0013 decreased with decreasing inoculum concentration. Suppression of Fusarium wilt diseases by root colonizing fungi with several mechanisms have been reported (Hossain *et al.* 2007, Narisawa *et al.* 2000, Meera *et al*. 1995).

Table 2. Effect of inoculum concentration of Penicillium sp. EU0013 on the disease severity caused by F. oxysporum f.sp. lycopersici CU1 to tomato plants and F. oxysporum f.sp. conglutinans K124F to cabbage plants.

EU0013: Pathogen (conidia/g of potting mix)	0:0	10^4:0	0:10^5	10^2: 10^5	10^3: 10^5	10^4: 10^5	10^5: 10^5	10^6: 10^5
Disease severity (%)[A] of tomato wilt	0	0	72.4 (e)	39.0 (d) (46)	33.1 (cd) (54)	26.4(bc) (64)	21.1 (ab) (71)	16.9(a) (78)
Disease severity (%)[A] of cabbage wilt	0	0	77.3 (e)	52.8 (d) (32)	44.1 (c) (43)	31.1(b) (60)	22.3 (a) (70)	19.9(a) (74)

Fisher's LSD for tomato wilt at (P <0.05) = 8.30; Fisher's LSD for cabbage wilt at(P <0.05) = 9.97

Inocula of both fungi were added as a water drench. Statistical comparisons between treatments were performed by F test (P <0.05). Letters in common in parenthesis indicate a lack of significant difference (comparisons are valid within each row).

[A]Disease severity was assessed as proportion of leaves with symptoms (yellowing and wilting) compared with the total number of leaves (n=9) at 40 days post-inoculation with the pathogen. Numbers in parenthesis indicate % disease reduction compared to pathogen inoculated plants alone.

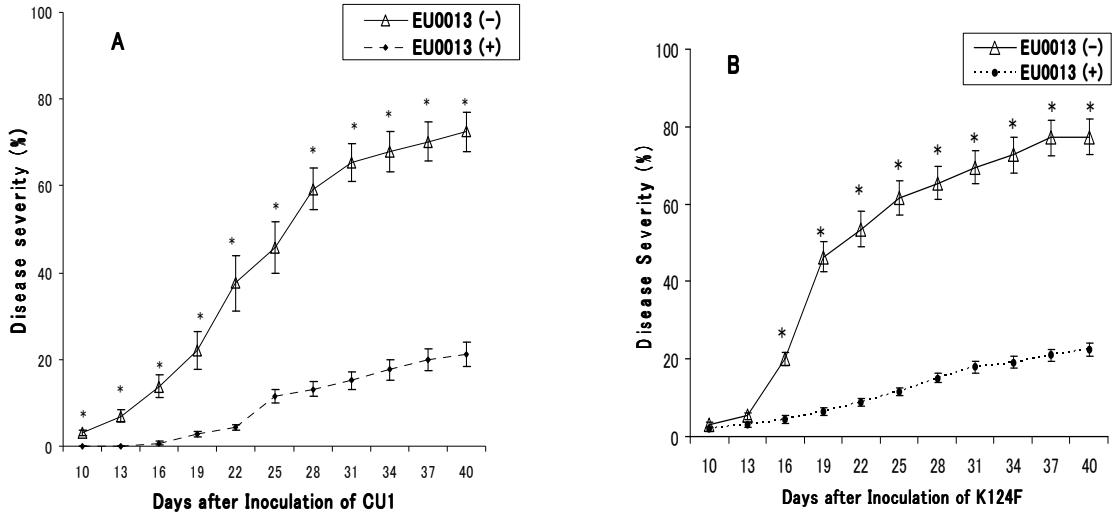

Figure 1. Effect of EU0013 on the development of Fusarium wilt diseases. (A) Tomato cultivar Oogatahukuju inoculated with *Fusarium oxysporum* f.sp. *lycopersici* CU1. Disease progress curves in tomato plants grown on potting mix in the absence (-) or presence (+) of 10^5 conidia /g of potting mix of EU0013. (B) Cabbage cultivar Shikidori inoculated with *Fusarium oxysporum*. f.sp. *conglutinans* K124F. Mean values that were statistically different at each time point ($P \leq 0.01$) are indicated by an asterisk. Bars indicate standard errors of means.

Table 3. Effect of inoculum concentration of *Penicillium* sp. EU0013 on the colonization of tomato and cabbage roots at 40 days post inoculation.

EU0013: Pathogen (conidia/g of potting mix)	10^4:0	10^2: 10^5	10^3: 10^5	10^4: 10^5	10^5: 10^5	10^6: 10^5
EU0013 recovery from tomato roots (%) [a]	81	39	45	53	58	64
EU0013 recovery from cabbage roots (%) [a]	79	36	41	45	55	60

[A]EU0013 recovery from roots tissues of inoculated seedlings= Number of EU0013 recovered roots segments/number of total roots segments incubated (n=30).

Conclusions

This study reports the biocontrol potential of a novel root-colonizing fungus, *Penicillium* sp. EU0013 against soil-borne Fusarium wilt diseases of tomato and cabbage under controlled environmental conditions. Prior application of EU0013 effectively reduced the development of Fusarium wilt in tomato and cabbage.

Acknowledgments

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Plant type differently promotes the arbuscular mycorrhizal fungi biodiversity in their rhizospheres after revegetation of a degraded, semiarid land.

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Abstract
The diversity of arbuscular mycorrhizal fungi (AMF) and their association with distinct plants species is crucial information in the early stages of revegetation procedures since the AMF roots colonization plays an important role improving plant establishment and growth. We carried out a study where we analyze the AMF community composition in the roots of *Ephedra fragilis*, *Rhamnus lycioides*, *Pistacia lentiscus* and *Retama sphaerocarpa* fourteen months after revegetation in a Mediterranean semiarid degraded area of southeast Spain in order to verify whether different plant species can variably promote the diversity of AM fungi in their rhizospheres after planted. We amplified a portion of 795 bases pairs of the small-subunit ribosomal DNA by means of nested PCR which was subjected subsequently to cloning, sequencing and phylogenetic analyses. Eight fungal sequence types belonging to *Glomus* group A and B and to the genus *Paraglomus* were identified. The different plant species had different AM fungal community composition. Thus, *R. lycioides* harbored the highest number of fungal types while as *E. fragilis* was colonized only by two fungal types specific for this plant species. *P. lentiscus* and *R. sphaerocarpa* harbored each one three fungal types and two of them were shared. All AMF sequence types were found in the natural soil. These results show that one effective tool to restore degraded lands is an increase in the number of plant species used, which would increase the AMF diversity in the soil and thus the below-ground, positive interactions.

Key Words
Mycorrhizal fungi, rhizosphere, revegetation, functional diversity, semiarid land.

Introduction
Several studies carried out in degraded semiarid Mediterranean areas showed that different shrubs representative of these areas differed in their ability to enhance the development of mycorrhizal propagules in the soil (Azcón-Aguilar *et al*. 2003; Caravaca *et al*. 2005), but whether these differences in the amount of AM fungal propagules correspond with variations in the AMF diversity is still unknown. There is increasing ecological interest in the diversity of AM fungi present in roots in field conditions, particularly with respect to revegetation programmes for degraded ecosystem using autochthonous shrubs, since the most functional species of AM fungi can serve as a source of inoculum for subsequent use in revegetation and/or recovery programmes for degraded soils. In this regard, the selection of plant species able to promote a high AMF biodiversity in their rhizosphere is an important point in the restoration of these difficult sites. In the present study, we used AMF-specific PCR primers, developed recently by Lee *et al*. (2008), to analyse the natural AMF communities which participate in the early colonisation of the roots of four shrub species after revegetation. These plants belong to the natural succession in semiarid Mediterranean ecosystems: *Ephedra fragilis* Desf., *Rhamnus lycioides* L., *Pistacia lentiscus* L. and *Retama sphaerocarpa* (L.) Boiss. The objective of this study was therefore to ascertain whether different plant species promote differently the diversity of AM fungi in their rhizospheres when planted in a semiarid degraded soil.

Methods
Study Site
The experimental area was located in Murcia (Southeastern Spain) (coordinates 38º12' N, 1º13' W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000mm. The mean annual temperature is 19.2 ºC with absence of frost period. The soil in the experimental area is a Typic Torriorthent (SSS 2006), very little developed with a low organic matter content and a silty clay texture that facilitates the degradation of soil structure.

Experimental Design and Sampling
The experiment was conducted using a randomized factorial design with six replication blocks. The

following plant species were selected: *Ephedra fragilis* (Desf.), *Rhamnus lycioides* L., *Pistacia lentiscus* L. and *Retama sphaerocarpa* (L.) Boiss. Once germinated, seedlings were transplanted into the growth substrate, consisting of 1 peat and cocopeat (1:1, v/v) autoclaved (60 min, 120º) in order to avoid any mycorrhizal propagules. They were grown with watering for 8 months under nursery conditions. In early April 2007, an area of 1.200 m^2 was selected to carry out the plantation. 30 seedlings of each plant species (5 per replication block) were planted in individual holes, separated between them at least 1m. The experiment was carried out under strictly natural conditions, without any watering or fertilizer treatments.
Fourteen months after plantation six plants belonging to each of the four selected species (one per replication block) were sampled (a total of 24 plants). Six samples of soil (one per replication block) of 500 g each were also randomly collected at 20-40 cm depths.

Roots and soil DNA extraction and PCR
For each sample, total DNA was extracted from root material (representing approx. 5-8 cm root
21 length) using a DNeasy plant mini Kit. For each of the six soil samples, genomic DNA was extracted from 0.5 g of soil using a FastDNATM 26 Spin kit for soil according to the recommendations of the manufacturer (Q-BIOgene, Heidelberg, Germany). Partial ribosomal small subunit (SSU) DNA fragments were amplified using nested PCR with the universal eukaryotic primers NS1 and NS4 (White *et al*. 1990). Several dilutions were used as template DNA in a second PCR reaction performed using the specific primers AML1 and AML2 (Lee *et al*. 2008).

Cloning and sequencing
The PCR products were purified using a Gel extraction Kit (Qiagen) cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* (Xl1 blue). Forty putative positive transformants were screened in each resulting SSU rRNA gene library, using 0.7 unit of RedTaq DNA polymerase (Sigma) and a re-amplification with AML1 and AML2 primers with the same conditions described above. Product quality and size were checked in agarose gels as described above. All clones having inserts of the correct size in each library were

Results
Twenty of the 24 root samples extracted and five of the six soil samples generated PCR products of the expected band of 795 bps, which were used for cloning and creating a clone library. From the 25 clone libraries, a total of 1000 clones were screened by PCR (on average, 40 clones were analysed per library); out of these, a total of 267 clones contained the SSU rRNA gene fragment and subsequently all clones were sequenced. The BLAST search revealed that 176 sequences (65.9%) had a high degree of similarity to sequences from taxa belonging to the phylum *Glomeromycota*, while the remaining sequences (34.1%) were similar to sequences from plant species. After Neighbour-joining (NJ) analyse of the sequences, eight AMF sequence types grouped in the *Glomeraceae* and *Paraglomeraceae* families could be distinguished on the basis of bootstrap values ≥ 77% (Figure 1). Six AMF sequence types belonged to *Glomus* group A, which was the group represented most frequently, one sequence type belonged to *Glomus* group B and one belonged to *Paraglomus*. Only two AMF sequences types identified in this study showed high similarity to sequences of previously-known glomalean species: Glo G1 clustered together with *Glomus lamellosum* and Glo G5 showed high similarity to the species complex *Glomus intraradices*/*Glomus fasciculatum*. Glo G 2b and Glo G4 showed high similarity to previously-described, root-derived sequences in GeneBank belonging to unknown glomalean species. Pa 1, although forming a different clade with a high bootstrap value (100%), showed high homology (95%) with *Paraglomus occultum*. The rest of the sequences (Glo 2a, Glo 2c and Glo G3) received strong support in the phylogenetic analysis but did not seem to be related to any sequences of AM fungi in the database (Figure 1). *R. lycioides* had the highest AMF richness, hosting four of the eight AM fungal types found in this study (Figure 2); also, this shrub showed the highest Shannon-Weaver diversity index (H'=1.15). *R. sphaerocarpa* and *P. lentiscus* harboured three AMF sequence types. The AMF communities of *E. fragilis* had the lowest diversity (H'= 0.56), with the lowest number of AMF sequence types. The sequence type Glo G2b was the most widespread and accounted for 41% of the AMF sequences detected. The eight fungal types were identified in the sequences derived from the soil samples (Figure 2), Glo G5 and Glo G2b being present in the highest proportion.

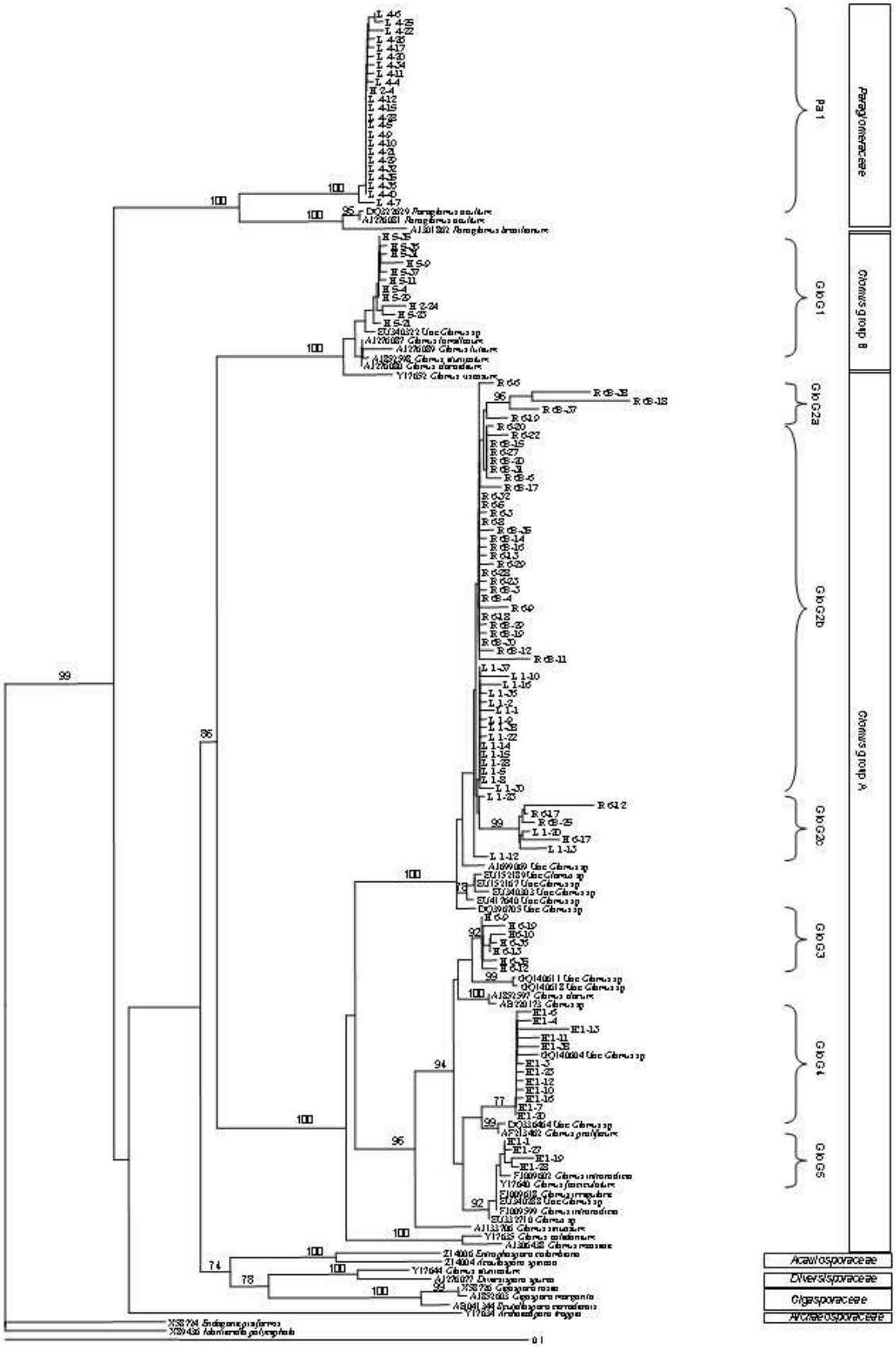

Figure 1. Neighbour-Joining (NJ) phylogenetic tree showing AM fungal sequences isolated from roots of *R. sphaerocarpa*, *R. lycioides*, *P. lentiscus* and *E. fragilis* and reference sequences from GeneBank. All bootstrap values > 75% are shown (100 replicates). Sequences obtained in the present study are shown in bold type. They are labelled with the host plant from which they were obtained (L= *P. lentiscus*, H= *R. lycioides,* R= *R. sphaerocarpa,* and E= *E. fragilis*) and the clone identity number. Group identifiers (for example Glo G1) are AM fungal sequences types found in our study. *Endogone pisiformis* and *Mortierella polycephala* were used as out-groups.

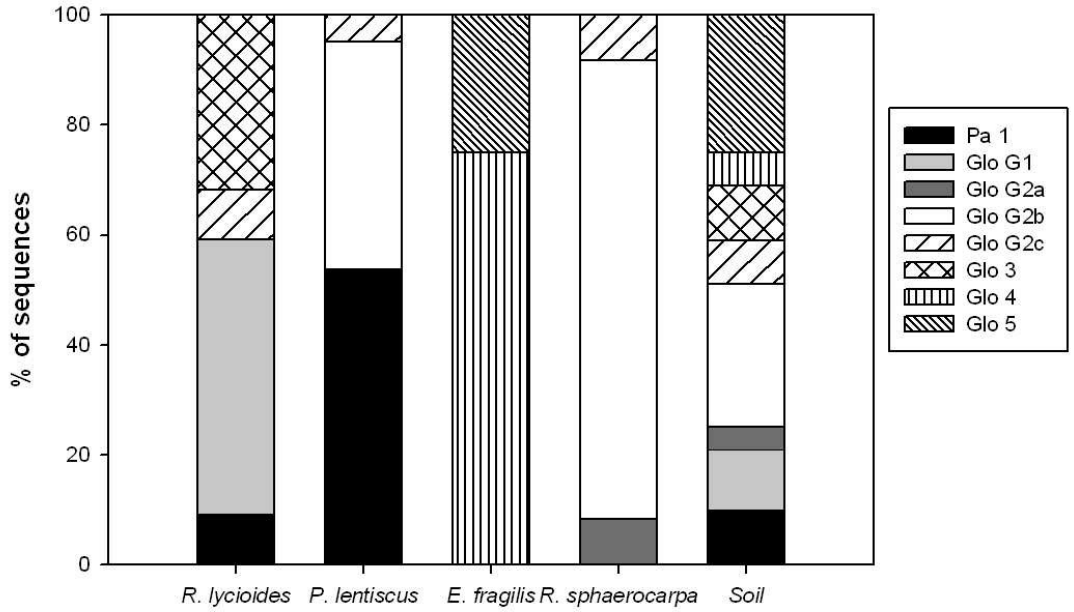

Figure 2. Bar plot showing the relative abundance of the different AM fungal types observed in *R. sphaerocarpa*, *R. lycioides*, *P. lentiscus* and *E. fragilis* roots and soil.

Conclusion

Ecosystems that have been degraded contain fewer AMF isolates. As we have demonstrated, the presence of different plant species results in the formation of mycorrhizae by different AM fungal species which "select" their host. In this way, a new fungal community 1 is created, as in a natural ecosystem where plant communities are associated with communities of AMF (Klironomos *et al*. 2000). In conclusion, this study shows that in revegetation programmes the AMF diversity is different depending on the host plant. Therefore, one effective tool to restore degraded lands is an increase in the number of plant species used, which would increase the AMF diversity in the soil and thus the below-ground, positive interactions.

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Characterization of copper biosorption and bioreduction by copper resistant bacteria isolated from a vineyard soil

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Abstract
This study presents factors affecting copper bioreduction and biosorption by a highly copper resistant monoculture of *Pseudomonas putida* strain NA and copper bioremoval from soil. Seven bacteria resistant to high concentration of Cu(II) were isolated from enrichment cultures of vineyard soils and mining wastes. Culture parameters influencing copper bioreduction and biosorption by one monoculture isolate were studied. The isolate was identified by 16S rRNA gene sequence analysis as a *Pseudomonas putida* strain NA (98% similarity). The optimal temperature for growth was 30ºC and bioremoval (bioreduction and biosorption) of Cu(II) was maximal at 35ºC. Considerable growth of the isolate was observed between pH 5.0 and 8.0 with the highest growth and biosorption recorded at pH 6.0. Maximal bioreduction was observed at pH 5.0. *Pseudomonas putida* strain NA removed more than 110 mg/L Cu(II) in water within 24 h through bioreduction and biosorption. More than 23 mg/L of Cu(II) was biologically reduced in 24h at an initial concentration of 100 mg/L. In 12 h, 20 mg/L of Cu(II) was removed through biosorption. Results indicate a great potential for use of *Pseudomonas putida* NA for bioremoval of copper from water and soil.

Keywords: Copper (II) resistant bacteria, biosorption, bioreduction, copper bioleaching

Introduction
Copper pollution of the environment occurs via addition of contaminated waste, mineral fertilizers and pesticides in crop production. In vineyards, sprays of various formulations containing copper as the active ingredient are used to control fungal diseases, including mildew, leaf spots and blights (Mirlean *et al.* 2007). Wastes from copper mining areas containing high concentration of copper are also major source of copper pollution of adjacent environments. High concentration of copper decreases the population of normal soil organisms and promotes microbial resistance to copper in contaminated environments (Atlas and Bartha 1997). Bioremediation is an important tool for environmental remediation of heavy metals (Okeke 2008). Biosorption and bioreduction of contaminants are effective bioremediation processes for removal of copper and other toxic heavy metals from the environment. Hence there is increasing interest in copper bioremoval by biosorption in aqueous media. Studies on copper bioremoval are not limited to bacterial isolates; the algae *Gelidium* has been employed for copper biosorption from industrial effluents (Villar *et al.* 2008, 2009). Bioreduction of copper Cu(II) to Cu(I) is catalyzed by copper-reductase. Reduction of Cu(II) to Cu(I) enhances copper mobility and consequently availability of copper to cell wall ATPases (Whiteley and Lee 2006). ATPases have affinity to Cu(I) and rapidly pumps it into the cell promoting copper biosorption and bioremoval from aquatic and terrestrial environments. We present copper bioreduction and biosorption by a highly copper resistant monoculture, *Psuedomonas* sp strain NA, isolated from vineyard soil contaminated with copper. We examined the environmental factors influencing copper bioreduction and biosorption by the monoculture isolate. Furthermore we studied the capacity of the monoculture isolate to remove copper from soil.

Materials and methods
A vineyard soil sample was collected from EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) experimental station, Bento Gonçalves, RS, Brazil. The isolate more efficient in copper biosorption and bioreduction was tested and used to characterization of environmental analysis. The isolate was identified by 16S rRNA gene sequence analysis as a *Pseudomonas putida* strain NA (98% similarity). It was studied the Cu(II)-resistance, Cu(II) biosorption and Cu(II) reduction in different pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), time course (0, 2, 4, 6, 8, 10, 12 and 24 h), copper concentrations (0, 50, 100, 150, 200, 250 and 300 mg/L) and temperature (20, 25, 30, 35 and 40ºC), and copper bioleaching from soil contaminated with 500 mg/kg of copper as ($CuSO_4.5H_2O$) after 6 days leached. Total copper was analyzed using an atomic absorption spectrometer. Aliquots of culture supernatant (200 µL aliquots) were diluted 20 times and injected into the

atomic absorption spectrometer. Copper biosorption was calculated as the difference in total copper added to the medium and remaining total copper in the medium after different microbial treatments. (CuBiosor = CuTotal added – CuTotal after growth). Copper reduction was quantified by measuring monovalent copper complex with 1mM neocuproine hydrochloride (Smith and McCurdy, 1952).

Results

Cu(II) bioreduction dynamics and their relationship to biomass development and biosorption is presented in Figure 1. Isolate NA grew slowly for the first 8 hours and thereafter grew rapidly until 24 h. An exponential growth pattern was observed from 6 hours to 24 h incubation. Copper bioreduction was rapid in 12 h and continued to increase over the 24 h incubation time. Approximately 20 mg/L and 23 mg/L copper were reduced after 12 and 24 h respectively. Copper biosorption profile for isolate NA similarly increased with biomass development during the 24 hours of incubation. After 12 h and 24 h, 23.33 mg/L and 21.66 mg/L copper were respectively bioremoved.

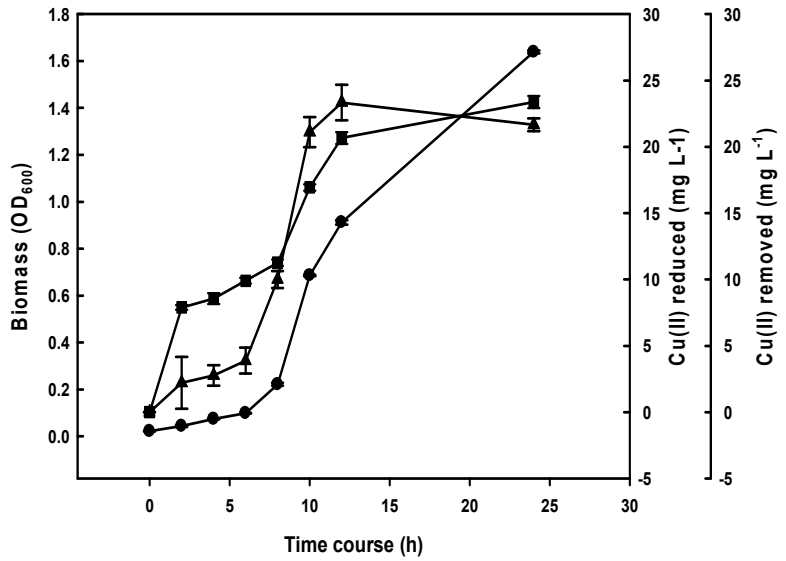

Figure 1. Time course of Cu(II) resistance (●), Cu(II) reduction (■), and Cu(II) bioremoval (▲) for Cu(II) resistant isolate NA in TSB medium contaminated with 100 mg/L of $CuSO_4$ and incubated at 30ºC for 24 hours. Error bars are standard errors of the means of 3 replicates.

The effect of pH on Cu(II) resistance, bioreduction and biosorption by NA is summarized in Table 1. Significant copper bioreduction occurred at initial pH range of 5.0 to 7.0 with optimum at pH 5.0 (31.04 mg/L of Cu(II) reduced in 24 h). The isolate substantially removed Cu(II) in the initial pH range of 5.0 to 9.0 and maximal removal occurred at pH 6.0 (26.25 mg/L of Cu(II) removed in 24 h). Substantial growth of the isolate was observed between pH 5.0 and 8.0, and the maximal growth occurred at pH 6.0. A slight change in initial pH occurred with growth of the isolates but it was not substantial. Little or no growth of the isolate was observed at pH 4.0 and no copper reduction was observed at pH 4.0.

Table 1. Effect of pH on Cu(II) resistance, bioreduction and bioremoval by isolate NA

Initial pH	Biomass OD_{600nm}	Cu(II) reduced ---------- mg/L ----------	Cu(II) removed	Final pH
4.00	0.069±0.001	00.00±0.001	2.29±0.481	3.79±0.057
5.00	1.847±0.029	31.04±1.042	22.92±0.524	5.59±0.009
6.00	1.866±0.009	20.74±0.228	26.25±0.208	6.45±0.032
7.00	1.639±0.006	16.93±0.327	21.67±0.481	7.26±0.011
8.00	1.189±0.003	7.34±0.208	21.25±1.156	7.96±0.013
9.00	0.745±0.007	4.85±0.097	24.79±1.069	8.74±0.009

Values are the means ± SD (n-1) of 3 replicates.

The relationship between Cu(II) concentration and biomass development are presented in Table 2. There was an inverse relationship between growth of NA and copper concentration in medium. Rates of copper

reduction and biosorption increased with increase in copper concentration to about 111.87 mg/L and 111.25 mg/L respectively. Isolate NA displayed strong bioremoval of Cu(II) with as much as 220 mg/L removed at the highest concentration.

Table 2. Effect of Cu(II) concentration on Cu(II) resistance (A), Cu(II) reduction (B), and Cu(II) bioremoval (C) in a TSB medium for 24 hours at 30ºC.

Cu(II) Concentration	Biomass -------- OD_{600} --------	Cu(II) Reduction ---------- mg/L ----------	Cu(II) Bioremoval ----------
0	1.45±0.0025*	0.00±0.0100	0.00±0.0010
50	1.34±0.0002	17.83±0.3205	19.58±1.3889
100	1.26±0.0020	33.18±0.1715	37.08±6.5972
150	1.20±0.0001	45.80±0.3267	48.33±0.3472
200	1.14±0.0014	62.35±9.3750	72.50±9.3750
250	1.05±0.0032	79.07±1.9506	90.42±2.4306
300	0.99±0.0002	111.85±15.727	111.25±19.791

Values are the means ± SD (n-1) of 3 replicates.

Temperature profile for Cu(II) bioreduction and bioremoval by sorption is presented in Figures 2A and 2B respectively. Growth of isolate NA was substantial between 20 and 35ºC. Optimal growth of the isolate in copper medium occurred at 30ºC. Cu(II) was maximally reduced and removed at 35ºC (Figures 2A and B). Isolate NA removed high copper concentrations between 30 and 35ºC. At 35 ºC more than 41 mg/L Cu(II) was removed.

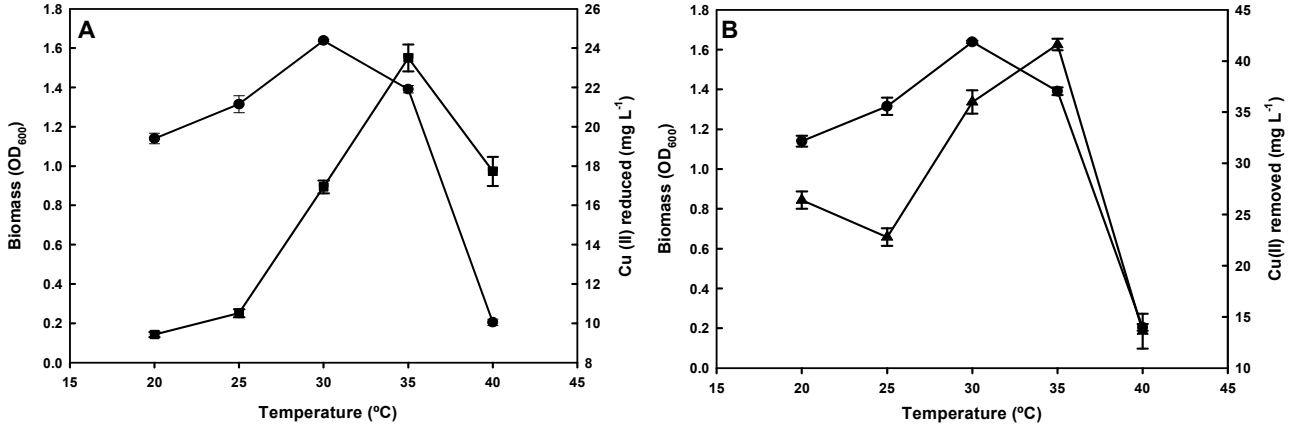

Figure 2. Effect of temperature on A: Cu(II) reduction (■), and B: Cu(II) bioremoval (▲), in TSB culture of isolate NA contaminated with 100 mg/L of Cu(II) and incubated for 24 hours. Biomass development profile (●). Error bars are standard errors of the means of 3 replicates.

Figure 3 shows copper bioremoval and biomass in leachate from copper contaminated soil inoculated with isolate NA suspension. In the control treated with distilled water cell density decreased slightly after 2 days and thereafter stabilized. Copper bioleaching in soil treated with distilled water (control) gradually increased during the course of the treatment. In soil cultures of isolate NA, biomass increased exponentially reaching maximum on day 4 and thereafter declined. Copper bioleaching from copper contaminated soil treated with isolate NA, rapidly increased during the course of the treatment and was significantly higher than in the control. Total copper bioleached from soil treated with isolate NA was over 18 mg/kg and only 8 mg/L in the control treated with distilled water.

Conclusions

Divalent copper is an essential micronutrient for living organisms but negatively impacts them at high concentrations. Consequently removal of high concentrations of copper from soils and aquatic environments is critical. Biological detoxification of pollutants is an attractive technology that is cost-effective and eco-friendly. In this study, *P. putida* NA displayed strong tolerance, bioreduction and bioremoval of Cu(II). Temperature, pH and pollutant concentration had marked influences on copper bioreduction and bioremoval capacity of *P. putida* NA. The isolate strongly promoted copper bioleaching in soil. The high Cu(II) tolerance, Cu(II) biosorption and bioreduction capacity of isolate NA as well as its stability in soil make it a

candidate organism for Cu(II) bioremoval in diverse complex environments contaminated with divalent copper.

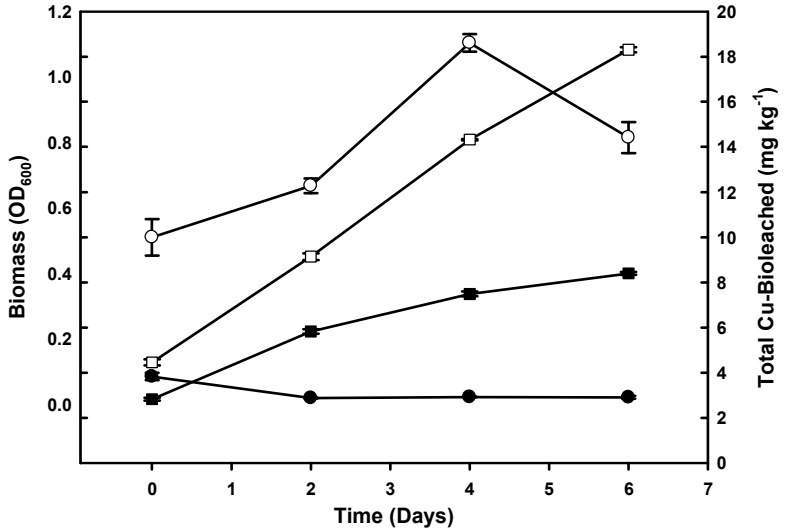

Figure 3. Bioleaching of Cu(II) from soil contaminated with 500 mg/kg. Cell density in soil treated with water (●), copper bioleached in soil treated with water (○), cell density in soil treated with *P. putida* cell suspension (■) and copper bioleached in soil treated with *P. putida* cell suspension (□). Error bars are standard error of the means.

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Colonization pattern of *gfp* tagged *Methylobacterium suomiens* on rice and tomato plant root and leaf surfaces

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Abstract
The localization of bacterial cells, pattern of colonization, and survival of *Methylobacterium suomiense* CBMB120 in the rhizosphere of rice and tomato plants were followed by confocal laser scanning, scanning electron microscopy, and selective plating. *M. suomiense* CBMB120 was tagged with green fluorescent protein (*gfp*), and inoculation was carried out through the seed source. The results clearly showed that the *gfp* marker is stably inherited and is expressed in planta allowing for easy visualization of *M. suomiense* CBMB120. The colonization differed in rice and tomato-intercellular colonization of surface-sterilized root sections was visible in tomato but not in rice. In both rice and tomato, the cells were visible in the substomatal chambers of leaves. Furthermore, the strain was able to compete with the indigenous microorganisms and persist in the rhizosphere of tomato and rice, assessed through dilution plating on selective media. The detailed ultrastructural study on the rhizosphere colonization by *Methylobacterium* put forth conclusively that *M. suomiense* CBMB120 colonize the roots and leaf surfaces of the plants studied and is transmitted to the aerial plant parts from the seed source.

Key Words
Methylobacterium, Green fluorescent protein, Conjugation, Colonization, Microscopy.

Introduction
Bacteria of the genus *Methylobacterium* possess one or more characteristics of plant-growth promoting bacteria (PGPB). Despite the beneficial effects, application of PGPB are often hampered in the field due to inconsistencies in the rhizosphere under different conditions. Therefore, a better understanding of the colonization pattern and the survival of introduced bacteria is a critical prerequisite (Compant *et al*. 2005). Strain CBMB120, a rhizosphere soil isolate from rice (*Oryza sativa* cv. Dong-jin) had plant growth promoting characteristics and the presence of acyl-homoserine lactone quorum sensing signal molecules for cell to cell communication has been also documented in this strain (Madhaiyan *et al*. 2006). In this study, *M. suomiense* CBMB120 was tagged with green fluorescent protein (*gfp*), and confocal laser scanning (CLSM) and scanning electron microscopy (SEM) were utilized to localize the bacterial cells in the rhizosphere in two different plant species. The persistence of the strain in the presence of indigenous microorganisms in rhizosphere was checked by selective plating.

Methods
Tagging of Methylobacterium with gfp
M. suomiense CBMB120 was tagged with *gfp* (CBMB120-*gfp*29) through triparental mating using *E. coli* S17-1 (pFAJ1820 - *Tn5gusA-gfp*). The stability of introduced marker was checked through several generations and through a starvation experiment at 4 °C. The presence of gfp was confirmed by PCR amplification using specific primers.

Plant experiments
Surface sterilized, pre-germinated rice and tomato seeds (7, 3 days for tomato and rice) after treatment with bacterial suspension (2-4 h) were transferred to phytatrays containing 200 g of sterile sand with 35-40 ml plant nutrient solution (Simons *et al*. 1996) or to multi-well trays filled with air-dried Wonjo-Mix bed soil, the vegetable raising growth medium. The phytatrays were covered with lid, sealed with parafilm. Two ml nutrient solution were added to each well in the tray at weekly intervals. The plants were grown under growth chamber conditions(25/20 °C; 70% humidity; 14/10 h).

Confocal laser Scanning and Scanning Electron Microscopy (CLSM; SEM)
Roots, leaves and hand cut transverse sections of sterile leaves and roots were mounted using Vectashield mounting medium under a coverslip. Microscopic observations were performed using Leica TCS SP2

confocal system equipped with an Ar ion laser (Gfp: excitation, 488 nm; emission filter BP 500-530). For SEM, samples were prepared according to Bozzola and Russell (1998), dried to critical point, coated with gold-palladium and visualized using a Hitachi S-2500C Scanning Electron Microscope.

Enumeration of bacterial population
The bacterial enumerations were carried out at 7, 14 and 21 days for rice and 14, 21 and 30 days for tomato. The rhizosphere soil population, the rhizoplane and endophytic colonization of roots and shoots was determined by serial dilution technique on 1/10 Tryptic soy agar (TSA) for total bacteria and AMS with antibiotics for the inoculated bacterial population.

Results
The strain CBMB120-*gfp*29 showed higher fluorescence and can be easily differentiated from the wild type and readily detected under CLSM (Figure 1). The PCR amplification with *gfp*29 specific primers as well as grown in antibiotic selective media revealed that integration of the mini-transposon into the bacterial chromosome was stable.

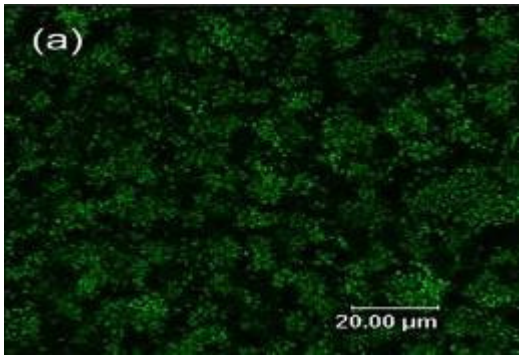

Figure 1. Confocal laser scanning microscopy of CBMB120-*gfp*29

Control plants without and with wild type CBMB120 inoculated plants observed by CLSM showed no fluorescent cells (Figure 2a). Sparsely distributed single cells of rod or circular shaped cells can be observed on the surface of rice roots (Figure 2b-c). A linear long string of closely associated cells can be observed throughout the entire length of the roots along the epidermal cell layers (Figure 2d). However, the transverse sections of rice roots obtained after surface sterilization showed no intercellular colonization. Numerous single cells were seen in the mesophyll and in the stomatal chambers of the leaves (Figure 2e-h). Similarly in tomato, fluorescent cells of CBMB120-*gfp*29 can be observed on tomato roots and leaves but the pattern of colonization remained different (Figure 3).

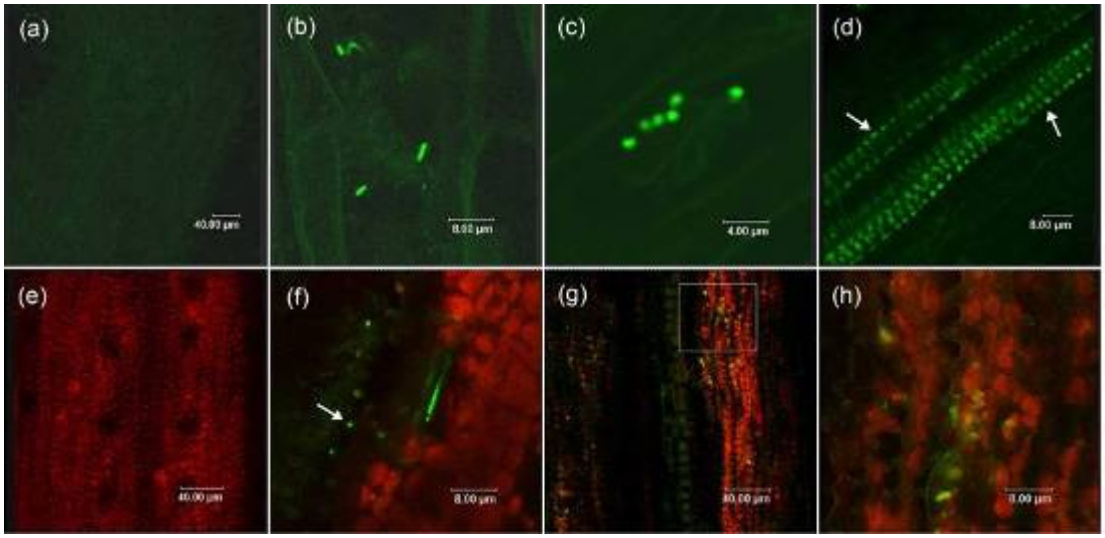

Figure 2. Colonization of rice roots and leaves by CBMB120 *gfp*29 observed through CLSM. (a) root of control plants; rod (b) and circular (c) shaped cells on the root surfaces of inoculated plants; (d) linear row of cells along the epidermal cells; (e) Leaves of control plants; (f-h) Single cells in the apoplastic region

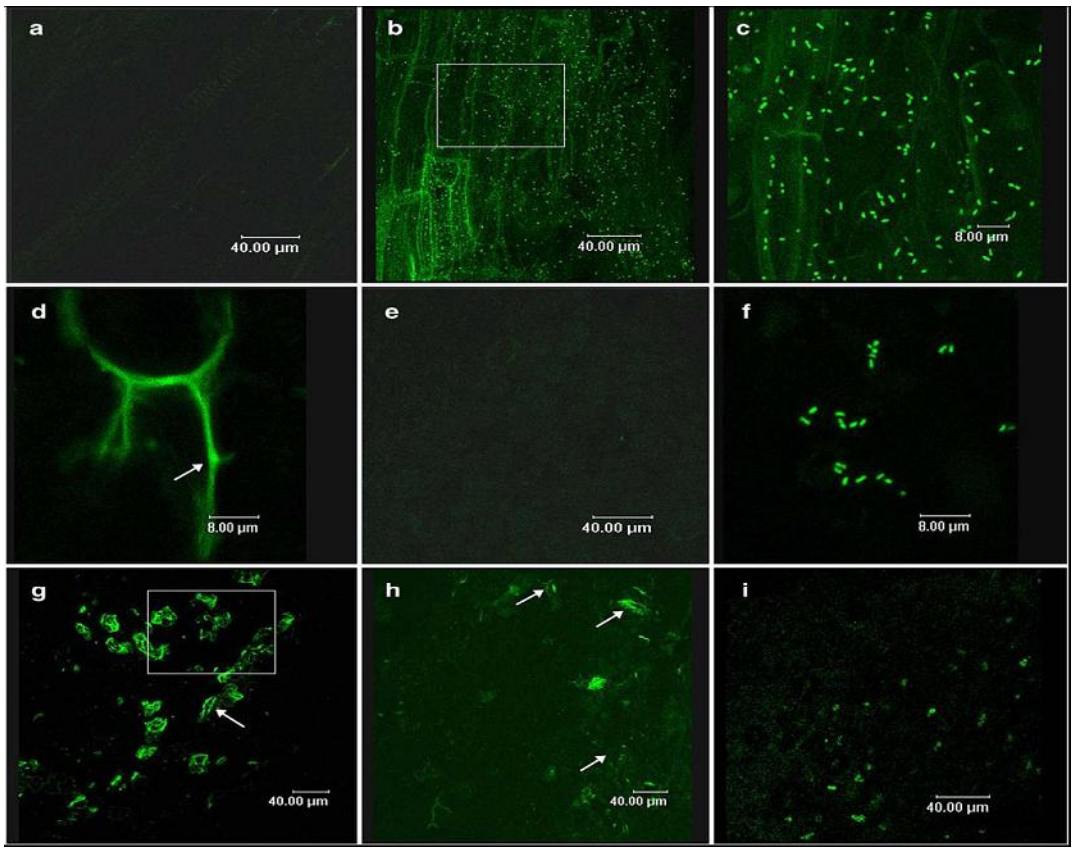

Figure 3. CLSM images showing the colonization pattern of CBMB120-*gfp*29 in tomato roots and leaves. (a) Root surface from uninoculated control. (b, c) Numerous rod-shaped cells can be observed colonizing the tomato root surface (c is an enlarged view of area pointed in b). (d) CBMB120-gfp29 penetration in to the intercellular spaces of root cortical cells can be identified by a strong fluorescence; (e–i) The colonization of leaf surfaces; (e) uninoculated control; (f) Presence of single cells on leaf surface; (g) Presence of CBMB120-gfp29 in the apoplast of leaves; (h) Cells in the substomatal chambers of leaves (i) The cells of CBMB120-gfp29 can also be observed in the surface-sterilized transverse section of tomato leaves

The results on the SEM observations for tomato is present in Figure 4. Numerous single and clusters of cells were dispersed throughout the length of the primary roots of tomato which often showed disrupted zones in roots (Figure 4a-c). Shortened, single cells were aggregated at the sites of emergence of secondary roots, which induced disruption of cortical and epidermal tissues (Figure 4d). The cells were unevenly distributed on the surface of tomato leaves with numerous shortened single cells lining the epidermal cells and clusters of single cells in the inter-cellular crevices of leaf surface and in the stomatal chambers (Figure 4e-h)

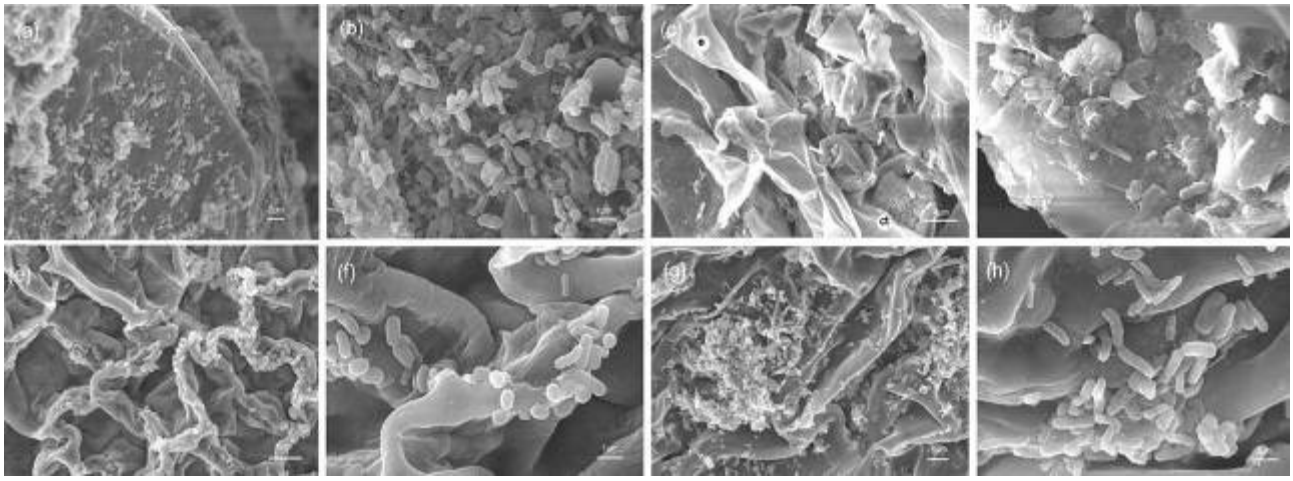

Figure 4. Distribution of *M. suomiense* CBMB120 on tomato roots and leaves of inoculated plants. e - Epidermis of primary root; ct - cortical tissues. The cells in the root surface showed adhesion filaments with in them and the rhizoplane.

Quantitative data on the survival of CBMB120-*gfp*29 in tomato and rice was obtained by selective plating on AMS with methanol supplemented with kanamycin (Table 1). No background growth was observed from non-inoculated control plants and the inoculated strain densities were higher in the rhizoplane than in the phylloplane. A considerable population was present in the rhizosphere soil also.

Table 1. Population of CBMB120-*gfp*29 in different parts of rice and tomato at three sampling periods

DAI	Population (log cfu g^{-1} sample)				
	Rhizosphere	Rhizoplane	Phylloplane	Root interior	Shoot interior
14*	3.95±0.14b	4.52±0.30a	3.24±0.14a	3.53±0.19a	3.26±0.15ba
21	4.81±0.12a	4.34±0.20a	3.25±0.14a	2.97±0.10b	3.30±0.12a
30	2.76±0.06c	3.16±0.09b	2.31±0.12b	1.12±0.07c	3.18±0.10b
LSD	0.18	0.41	0.05	0.25	0.09
7	3.98±0.16b	5.64±0.14a	3.25±0.14c	3.50±0.06c	2.87±0.16b
14	3.63±0.13c	5.57±0.21a	4.15±0.09a	4.59±0.11a	3.06±0.15a
21	4.06±0.15a	5.25±0.14b	3.76±0.21b	4.05±0.14b	2.47±0.21c
LSD	0.06	0.16	0.24	0.17	0.14

DAI- days after bacterial inoculation; * the days of bacterial enumeration. Within each column, values followed by the same letter are not statistically different at $P \leq 0.05$

Conclusion

M. suomiense CBMB120, a rhizosphere soil isolate colonize the roots and leaf surfaces of plants without host speciation. The strain is transmitted to the aerial plant parts from the seed source rather than from environmental sources and able to persist in the rhizosphere in the presence of indigenous microorganisms.

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Effect of biochar on arbuscular mycorrhizal colonisation, growth, P nutrition and leaf gas exchange of wheat and clover influenced by different water regimes

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Abstract
Biochar (biomass-derived carbon) can influence arbuscular mycorrhizal colonisation, plant growth, P nutrition and water relations but research is not conclusive. Therefore, a glasshouse experiment was conducted using wheat and clover with three water regimes, two biochar rates (0, 6 t/ha) with a series of harvests. Mycorrhizal colonisation was altered by water regimes. Shoot and root dry weight increased in well-watered compared to water-stressed conditions. Root length and colonised root length increased in the biochar treatment for wheat, especially in the well-watered treatment. Leaf gas exchange was higher in the presence of biochar indicating increased water availability to plants growing in biochar treated soil.

Key Words
Arbuscular mycorrhizal fungi, biochar, clover, growth, leaf gas exchange, wheat.

Introduction
Water deficit is considered one of the most important abiotic factors limiting plant growth and yield in many areas on earth; especially in the context of changing climates and agricultural areas expected to experience increased frequency of dry seasons. Several eco-physiological studies have demonstrated that the arbuscular mycorrhizal (AM) symbiosis can result in an altered rate of water movement into, through and out of the host plants, with the consequent effects on tissue hydration and plant physiology. It is now accepted that the contribution of AM symbiosis to plant drought tolerance is the result of accumulative physical, nutritional, physiological and cellular effects (Al-Karaki *et al.* 2004;Ruiz-Lozano 2003). Application of biochar (pyrolised biomass) to soil can result in significant responses by both plants and mycorrhizal fungi. Biochar has been shown to increase mycorrhizal root colonisation and create a microhabitat in soil (Saito 1990; Warnock *et al.* 2007). Based on recent research in Western Australia biochar may encourage colonisation by mycorrhizal fungi and improve plant water supply in drought risk environments (Blackwell *et al.* 2007). The aim of the present study was to determine (i) the effect of water regimes on mycorrhizal colonisation, plant growth, P nutrition and water relations of wheat and clover (ii) the interaction between biochar and water regimes on mycorrhizal colonisation and (iii) the effect of biochar on leaf gas exchange in relation to water availability.

Material and methods
The experiment was arranged in a factorial and randomized complete block design with six treatments and three replications. The treatments included three water regimes, two biochar levels, two crops and four harvests as bellow: water regimes: I1: 80% of F.C (well-watered); I2, 40% of F.C (water stressed), and I3, intermittently watered (periodic water stressed). Jarrah biochar was obtained from a 35 years old stockpile of metallurgical charcoal at the site of an iron foundry at Wundowie, WA. The pH of biochar was 4.8 and this acidity was unusual compared to the other biochars and applied at 0 and 6 t/ha. There were 3 water regimes × 2 biochars × 2 crops × 4 harvests ×3 replications (144 pots). Each pot contained 1 kg of field soil of sandy clay loam collected from Walkaway, WA amended with western mineral fertilser as a basal at 50 kg/ha. Five seeds of each wheat (*Triticum aestivum* L.var. Brookton) and subterranean clover (*Trifolium subterranean* L. var. Seaton Park) were sown and allowed to grow for 5 days after germination. The three most vigorous plants were retained in each pot and harvested 14, 24, 34 and 44 days after sowing. Data collected were: shoot and root dry weight; % root length colonised by AM fungi; total and colonised root length, phosphorus concentration in shoot, and leaf gas exchange such as photosynthesis, stomatal conductance, internal CO_2 concentration and transpiration. Leaf gas exchange was measured using a Li-Cor 6400 photosynthesis system (Li-Cor, Lincoln, NE, USA).

Results

AM colonisation increased significantly in the biochar treatment for wheat grown in well-watered and periodic water stressed treatments (Figure 1). Total plant root length and AM colonised root length increased significantly ($p \leq 0.05$) in the biochar treatment for wheat but only in colonised root length was increased for subterranean clover ($p \leq 0.05$) (Figure 1). The effect of water regimes on AMF colonisation, plant biomass, and root length were all significant ($p \leq 0.05$). The effect of biochar and water regimes on leaf gas exchange such as photosynthesis, stomatal conductance, internal CO_2 concentration and transpiration were significant ($p \leq 0.05$) (Figure 2).

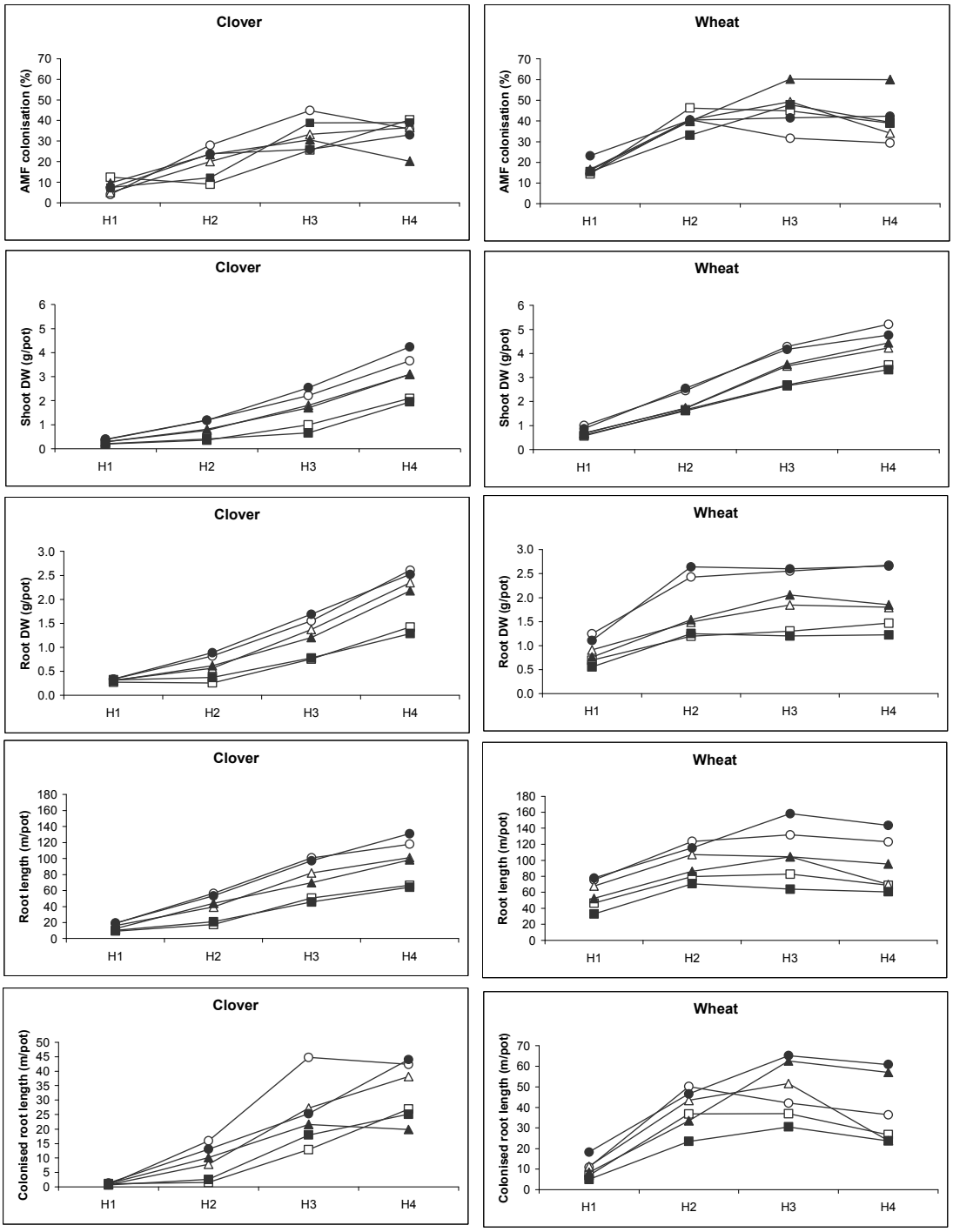

Figure 1. Effect of biochar and water regimes on mycorrhizal colonisation, plant biomass and root length of subterranean clover and wheat; O, well-watered; □, water-stressed and Δ, periodic water-stressed; biochar - closed symbol, no biochar – open symbol; H1-4 harvests.

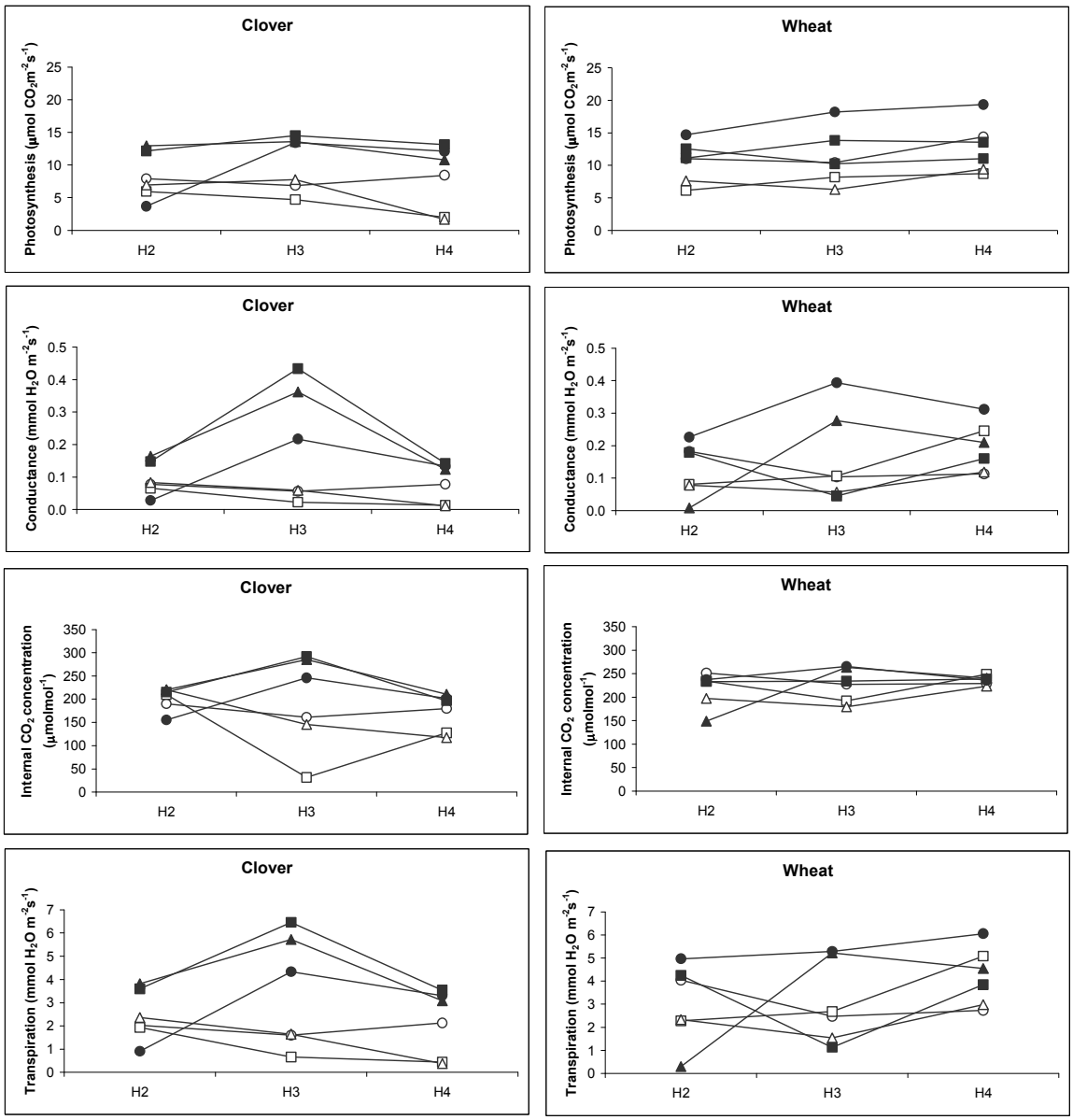

Figure 2. Effect of biochar and water regimes on leaf gas exchange of clover and wheat; O, well-watered; □, water-stressed and Δ, periodic water-stressed; biochar - closed symbol, no biochar – open symbol; H1-4 harvests.

Conclusion

The effect of biochar application on AM colonisation, plant growth, root length, P nutrition was positive, especially in wheat strongly influenced by different water regimes. Leaf gas exchange was significantly higher in biochar treated plants suggesting increased water availability in the rhizosphere of biochar treated plants. These data support the expectation that biochar application may assist water supply in a dry season in a field experiment with the same biochar, fertiliser regime and soil.

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Effect of different fertilization practices on soil microbial activities and community structure in volcanic ash citrus orchard soil

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Abstract
This study was performed to evaluate effects of different fertilization practices on soil microbial activities and community structure using soil enzyme activities, PLFA contents in volcanic ash citrus orchard soil. Urease activity was high in NPK+Compost treatment, July. Dehydrogenase activity was higher in 1/2NPK+Compost (4.3 ug TPF /g 24 /h) than other treatments in May. β-glucosidase activity was higher in May than in September. That showed significant in between treatments and ordered NPK>1/2 NPK+Compost >NPK+Compost>Compost>3NPK>Control. Soil basal respiration rate decreased gradually and that was not different significantly between treatments. Total PLFA contents were higher in NPK+Compost than in compost. Distribution ratio of soil microbial groups by PLFA profiles as biomarker showed that bacteria and actinomycetes increased and both fungi and mycorrizae decreased in July relative to March. Principal component analysis of the microbial community by PLFA pattern showed that PLFA profiles from NPK and NPK+Compost plots were different at the 3NPK plot in March. Our result showed that composition change in microbial community were affected by a fertilization effect and seasonable factor.

Key Words
Volcanic ash soil, citrus, PLFA, urease, nicrobial community.

Introduction
Environmental factors such as soil type, temperature, and moisture, application of organic and inorganic fertilizers play an important role in microbial activity. Soil microbial activity was low as phosphate is converted to non-available fractions easily in volcanic ash soil. The more inorganic fertilizer applied for more production of citrus fruit, the more soil pH values became low and microbial activity was decreased by soil acidity. Soil microbial activity could be used efficiently to evaluate soil health and quality. This study evaluated the effect of long-term application of compost and inorganic fertilizer on soil microbial activity using several parameter including soil enzyme activities, soil basal respiration rate, microbial biomass, phospholipid fatty acids (PLFA)contents.

Methods
Fertilization management Citrus (*Citrus unshiu* Marc.) have been managed according to the application rate of fertilizers and compost for 13 years in Andisol volcanic ash soil. Experiment plots were composed of six treatments such as Control, Compost (20 ton/ha), NPK (280-400-280 kg/ha), 1/2NPK+Compost (140-200-140 kg/ha+20 ton/ha), NPK+Compost (280-400-280 kg/ha +20 ton/ha), 3NPK (840-1200-840 kg/ha). Compost was applied as matured cow manure compost in March. Nitrogen and potassium fertilizers were applied in March, May, and October, which were 40, 30, 30% of yearly application rate, respectively and phosphate fertilizer applied at one time in March. Besides of fertilization management followed conventional management. Soil samples were taken early in March, May, July, September, 2007. Soil samples after sieving were stored immediately at 4℃ for soil enzyme activities and biomass N and at -20℃ for PLFA until analysis.

Analysis Dehydrogenase activity was measured by triphenylformazan method (Rossel and Tarradellas 1991). Urease (Tabatabai 1976) and β‐glucosidase (Garcia‐Gil *et al.* 2000) activities were measured by the THAM buffer method. Biomass N was measured by Ninhydrin method (Amato *et al.* 1988). PLFA analyzed with a GC-FID instrument after Bligh/Dyer first‐phase extraction (Bligh and Dyer, 1959). Soil respiration rate was measured at 3, 10, 30 days by the 0.1M NaOH absorption method (Cerhanova *et al.* 2006).

Results

Soil enzyme activities are shown in Table 1. Urease activity was higher in NPK+Compost than in control, but was not significantly in between treatments. Dehydrogenase activity was in the orders of 1/2NPK+Compost > Compost > NPK > NPK+Compos t> 3NPK > Control in May. β-glucosidase activity was higher in NPK (38.2) than in Control(17.5 ug PNP /g/h) in May. Results indicated that urease and dehydrogenase activities in volcanic ash soil were low and not significantly different according to application rate of fertilizer and compost. β-glucosidase activity in NPK was considered high as an effect of fallen leaves from the citrus. Total PLFA content was higher about 2 times in NPK+Compost (237.4) than in 3NPK (133.0 n mol /g) in May (Figure 1). This result considered as an effect of soil acidity because soil pH value was lower for higher application of fertilizer, 3NPK compared with NPK. Soil respiration rate was not significantly different in between treatments (Figure 2).

Table 1. Soil enzyme activities for different fertilization management practices.

Treatments	Dehydrogenase				β-glucosidase			
	March	May	July	September	March	May	July	September
	------ ug TPF /g 24/h ------				------ ug PNP /g/h ------			
Control	3.2±0.4†	2.4±0.4b††	2.7±0.6	4.3±1.2	21.2±3.5c	17.5±3.1b	21.4±1.3	7.0±0.9
Compost	5.1±3.2	3.6±0.8ab	3.2±0.5	7.2±3.2	34.4±5.2ab	30.3±10.2ab	30.0±5.5	13.9±5.2
1/2NPK+Compost	5.5±1.9	4.3±0.2a	4.2±1.3	10.3±5.9	37.8±2.4a	32.1±2.5a	33.2±2.8	13.7±6.4
NPK	4.5±1.2	3.5±1.6ab	4.3±2.8	5.6±3.8	36.5±3.4a	38.2±12.8a	37.2±12.2	14.6±4.0
NPK+Compost	4.5±1.3	3.1±0.5ab	3.4±1.6	7.5±6.7	29.2±2.7b	30.4±5.4ab	37.3±11.9	10.3±4.1
3NPK	3.9±0.8	2.8±0.8ab	2.5±0.4	3.6±0.0	37.4±6.0a	27.0±6.0ab	30.7±6.0	14.4±4.9

†Mean ± Standard Deviation.
†† DMRT at p=0.05 level.

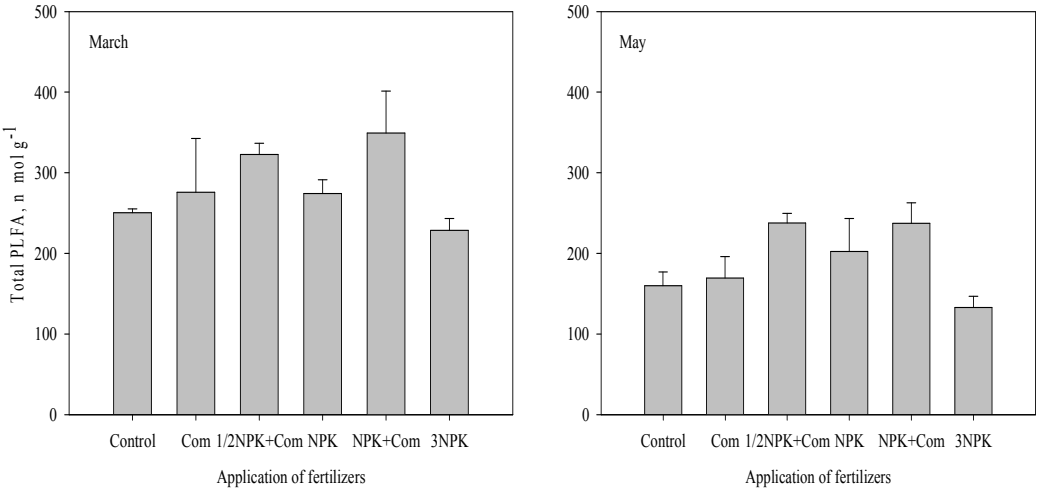

Figure 1. The amount of phospholipid fatty acid caused by different fertilization management practices.

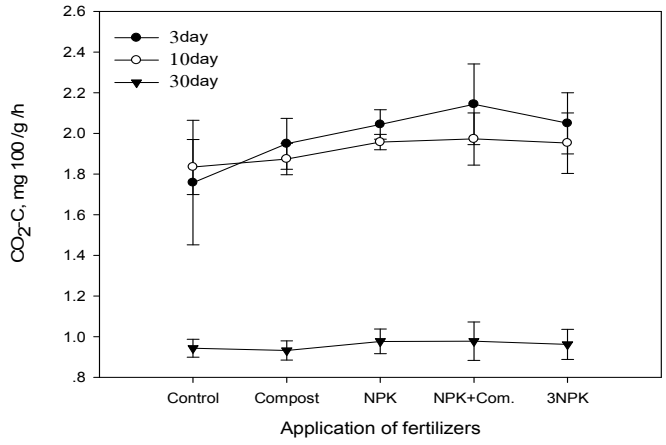

Figure 2. Soil respiration rate for different fertilization management practices for volcanic ash citrus orchard soil.

Conclusion

Urease and dehydrogenase activities in volcanic ash soil were low and not significantly different according to application rate of fertilizer and compost. β-glucosidase activity was higher in NPK (38.2) than in Control (17.5 ug PNP /g/h) soil in May. Soil basal respiration rate was not significantly different between treatments. Principal component analysis of microbial community by PLFA pattern showed that PLFA profiles from NPK and NPK+Compost plots were different at 3NPK plot in March. Our result showed that the microbial community was affected by fertilizer treatment effect and seasonal factors.

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Effect of soil properties on Pinot Noir vine vigour and root distribution in Tasmanian vineyards

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Abstract
Root distribution of *Vitis vinifera* cv Pinot Noir was described growing in 16 different soil variants within three vineyards in Tasmania, Australia.

Soil type was shown to affect the distribution of vine roots, with soil physical properties having the greatest influence. Roots responded to zones of soil weakness (such as cracks, sand in-fills and old root pathways), especially in coarsely structured soil conditions. In the absence of these features, high soil strength hindered root growth with 2000 kPa being a key threshold. The presence and frequency of these structures had a greater influence on root distribution than the overall soil strength. Soil chemical differences were less limiting and only caused restrictions to root growth at extreme levels.

Low vigour vines had a lower rooting depth (< 60 cm), and low total root numbers. Vines with high vigour had a considerable proportion of roots below 60 cm. High vine vigour did not always relate to high total root numbers suggesting that root efficiency and function is also important. Root distribution also generally followed water movement pathways, highlighting the importance of appropriate water management.

Key Words
Root distribution, soil, vine, *Vitis vinifera*, soil structure

Introduction
Pinot Noir is an increasingly important grape variety for cool climate wine production in Tasmania. The state has a very wide array of soil types used for production ranging from highly alkaline to acidic reaction trends and from reactive clay soils to loose sandy profiles. Soil depth, substrate conditions and water table heights are also highly variable. It has been noted that grapevine vigour and yield is highly varied both within and between vineyards. This study aimed to understand the soil component of vineyard variability. Investigations of vine root distribution were undertaken in different soil types and related to vine vigour and yield.

The findings of the study have implications for the expansion of the viticultural industry in Tasmania by identifying the most favorable soil conditions for high quality Pinot Noir production. The results also offer some useful insights for managing water and nutrient supply to vine root systems.

Methods
A total of 25 plots consisting of 12 vines each were established across three vineyards in Tasmania, Australia. These plots mainly consisted of *Vitis vinifera* cv Pinot Noir and covered 16 different soil variants.

Root distribution was recorded using the profile wall method outlined in Bohm (1979). Trenches were excavated to expose soil profiles and vine root distribution across two vines within each plot. Penetration resistance and root size frequency was recorded across the soil face using a 5 cm x 5 cm grid. The soil profile was described and sampled for chemical, physical and mineralogical analysis.

Vine parameters of bunch number, bunch weight, cane number and average cane weight were also recorded over three consecutive seasons to help derive overall yield and vigour characteristics for each plot.

Results
Soil type was shown to influence the distribution of vine roots, with differences in soil physical properties having the greatest affect. Soil chemical differences (salinity, exchange cations, pH and available P) were less limiting and only caused restrictions to root growth at extreme levels.

The total number of roots observed varied across the study, with high root numbers generally associated with high vine vigour. However, some vines had high vigour with relatively low total root numbers (Figure 1b). This suggests that root function and root efficiency has a greater influence on vine growth than total root number. It is assumed that water retention characteristics of each soil type play an important role, with soils that allowed easy access to moisture having high vine vigour with smaller root systems.

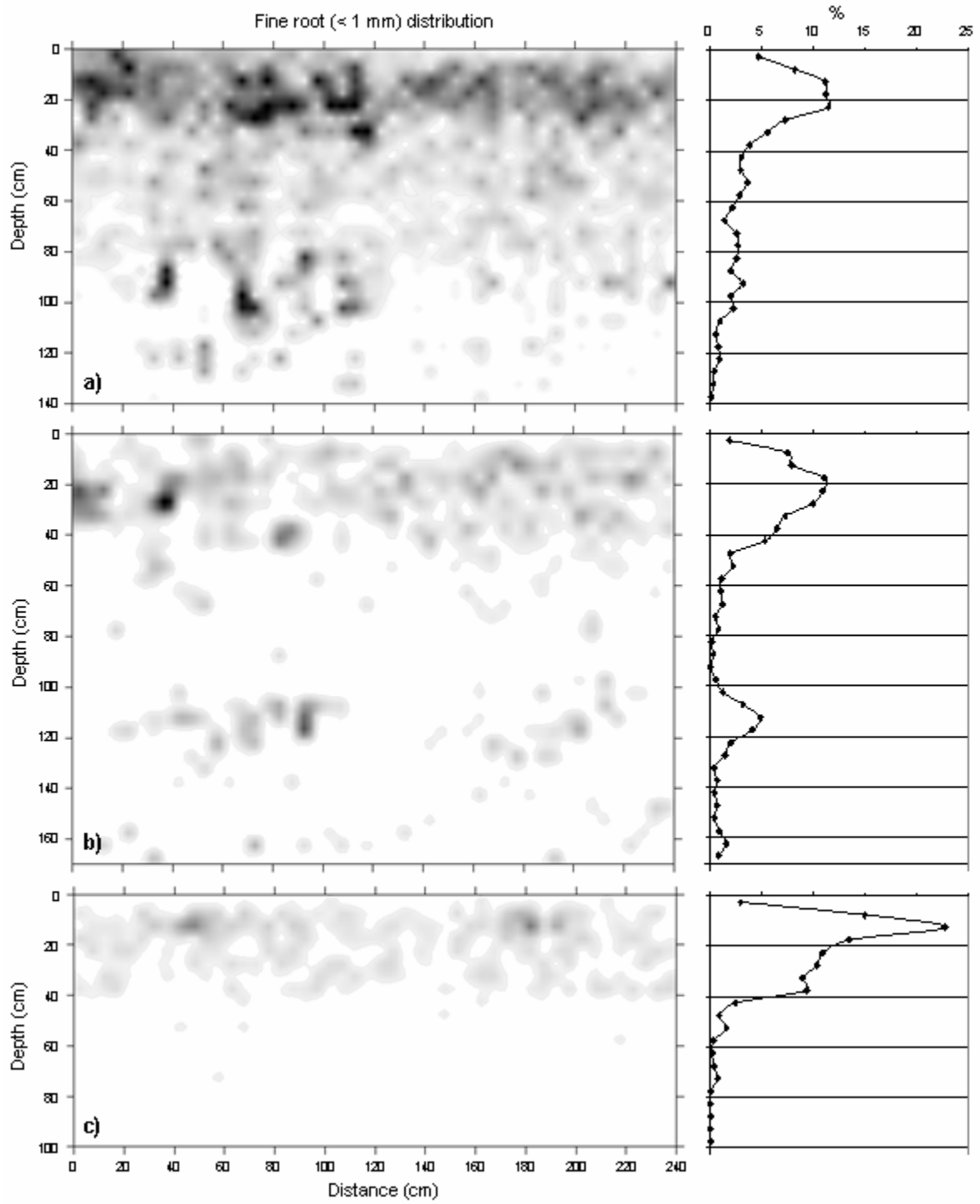

Figure 1. Examples of fine root (< 1 mm) distribution (shaded) and percentage root distribution with depth. a) extensive root distribution of high vigour vines with high root numbers. b) root distribution of high vigour vines with low root numbers and concentration of roots in subsoil. c) shallow root distribution of low vigour vines with low root numbers.

Independent of soil type, all vines had the highest percentage of root growth within 10 – 30 cm depth. Root growth was generally limited in the surface soil (0 – 10 cm) as well as at depths greater than 60 cm. Vines with high vigour had a high proportion of roots at depth (> 60 cm). Roots were either evenly spread through subsoil horizons (Figure 1a) or were concentrated at a specific depth (Figure 1b). Low vigour vines had

restricted rooting depth (< 60 cm) and low total root numbers (Figure 1c). Limited rooting depth was caused by shallow depth to impermeable layers (bedrock or massive subsoil), acid soil conditions (pH_w < 4.5) or shallow, saline water tables.

Root growth was reduced through soil with penetration resistance values greater than 2000 kPa. However, sites with high soil strength (> 2000 kPa) were still able to have extensive root growth if roots had access to cracks, sand in-fills or old root channels (Figure 2). This suggests that it is the void architecture of soil that is critical for root growth, rather than just soil strength.

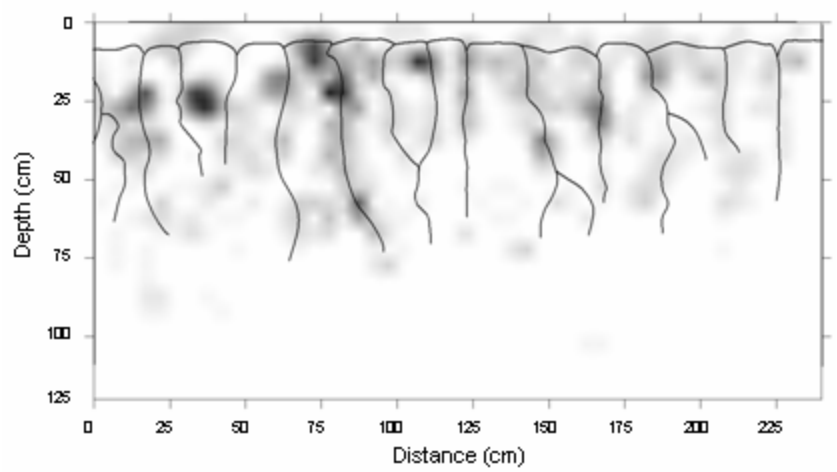

Figure 2. Fine root (< 1 mm) distribution (shaded) showing strong association with primary structure (–) in a soil with high soil strength (> 2000 kPa)

Conclusion

A wide range of root patterns and rooting depths were noted across the broad array of soil types observed. Peaks of root numbers occurred when favorable conditions were present, with soil structure having a greater influence than soil chemistry. Roots responded to cracking patterns and soil structure, especially in coarsely structured soil conditions. In the absence of cracks, high soil strength hindered root growth with 2000 kPa being a key threshold. Vigour was not always related to total root number suggesting root function is also significant. Root distribution also generally followed water movement pathways, highlighting the importance of appropriate water management.

Of the 16 soil variants observed, many showed favourable conditions for root growth with no one soil type or parent material that provides the 'ideal' medium for root growth. Moreover it is the physical conditions that the soil provides that have the greatest influence on root growth and vine vigour.

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Effects of legumes on arbuscular mycorrhizal colonisation and phosphorus uptake by the following wheat

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Abstract
It has been shown that biomass and P uptake in cereals following legumes are higher than in cereals following cereals. This may be due to soil chemical and biological changes induced by the legumes and/or their residues. However, the relative importance of legume pre-crops and their residues on P uptake and arbuscular mycorrhizal colonisation of wheat has not been investigated. The aim of the overall project is to separate the two effects by comparing the following treatments: legume pre-crops with or without residue incorporation and legume residues added without pre-growth of legumes in the soil on P uptake and AM colonisation of wheat. Here we present the results of three short experiments that provide important data for the main experiments.

Key Words
Legume residues; AM fungi; P uptake

Introduction

A number of studies have shown that biomass and P uptake in cereals following legumes are higher than in cereals following cereals (Armstrong *et al.* 1997; Asseng *et al.* 1998; Nuruzzaman *et al.* 2005). The positive effect of legumes to the following wheat may be due to the growth of legumes prior to wheat and/or due to nutrients released during decomposition of legume residue that are utilised by the subsequent wheat (Nuruzzaman *et al.* 2005). However, information about the effect of legumes as a pre-crop and their residues on P availability and P uptake is very limited.

In African soils, legume as a pre-crop also affects the biological properties: legume pre-crops result in earlier colonisation of cereal roots by AM fungi (Bagayoko *et al.* 2000). However, it is not known if such biological changes also occur in Australian soils and how AM fungal colonisation is linked to legumes as pre-crop and legume residue application.

The aim of this study is to determine the effect of legumes as a pre-crop and also their residues on colonisation of arbuscular mycorrhiza and P uptake by the subsequent wheat. To do so, three short experiments were carried out to provide basic information about the effects of available P concentration on AM colonisation and the relationship between additions of different legume residues on available P.

Methods

Experiment 1: Relationship between addition of inorganic P and available P in Monarto soil
The aim of this experiment was to determine the relationship between rate of inorganic P addition and available P. Inorganic P was added as KH_2PO_4 at 2.5 to 60 mg P/kg to soil from Monarto (South Australia) . The soil was incubated at 70% WHC for 5 days and then available P was determined as resin P.

Experiment 2: Available P and AM colonisation
The aim of this experiment was to investigate the relationship between P availability and AM colonisation in wheat. Wheat was grown in a soil with low available P (Monarto) over 6 weeks at 11 different rates of inorganic P addition (0, 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30, and 40 mgP/kg as KH_2PO_4). AM colonisation was determined after weeks 6 using gridline intersection method (Brundrett *et al.* 1996; Giovannetti and Mosse 1980).

Experiment 3: The relationship between legume residue addition and available P in soil
This experiment was conducted to investigate the relationship between rate of residue addition and available P. Four rates (0.5, 1, 1.5 and 2% w/w) and four types of residues (chickpea (CP), faba bean mature shoot (FMS), faba bean young shoot (FYS) and faba bean young root (FYR) were added to the soil and incubated for 5 days and the available P of the soil was determined.

Main experiment
Germinated seeds of wheat, faba bean, chickpea and white lupin will be sown at 2 cm depth and thinned after 2 weeks to 2 plants per pot; unplanted pots will serve as controls. After 8 weeks, three treatments will be imposed: to determine the effect of legumes as pre-crop and their residues, shoots of each legume will be removed, while roots will remain in the soil. In order to determine the pre-crop effect alone, both roots and shoots will be removed completely. To investigate the effect of root and shoot residues in absence of a pre-crop effect, shoot and root residues will be added to previously unplanted soil.

The following wheat will be planted immediately without a fallow period. During wheat growth, P availability in soils will be determined after 1, 2, 4 and 6 weeks. AM colonisation, wheat growth and P uptake will be assessed after 6 weeks.

Results
Experiment 1
Figure 1a shows that available P increased with increasing amount of inorganic P added. At 2.5 mg P/kg added P, only a small percentage (less than 20%) was recovered as available P. At 5 mg P/kg about 50% remained available. At higher rates of P addition, nearly all P remained available. This suggests that the buffering capacity of the soil is saturated at P addition ≥15 mg/kg.

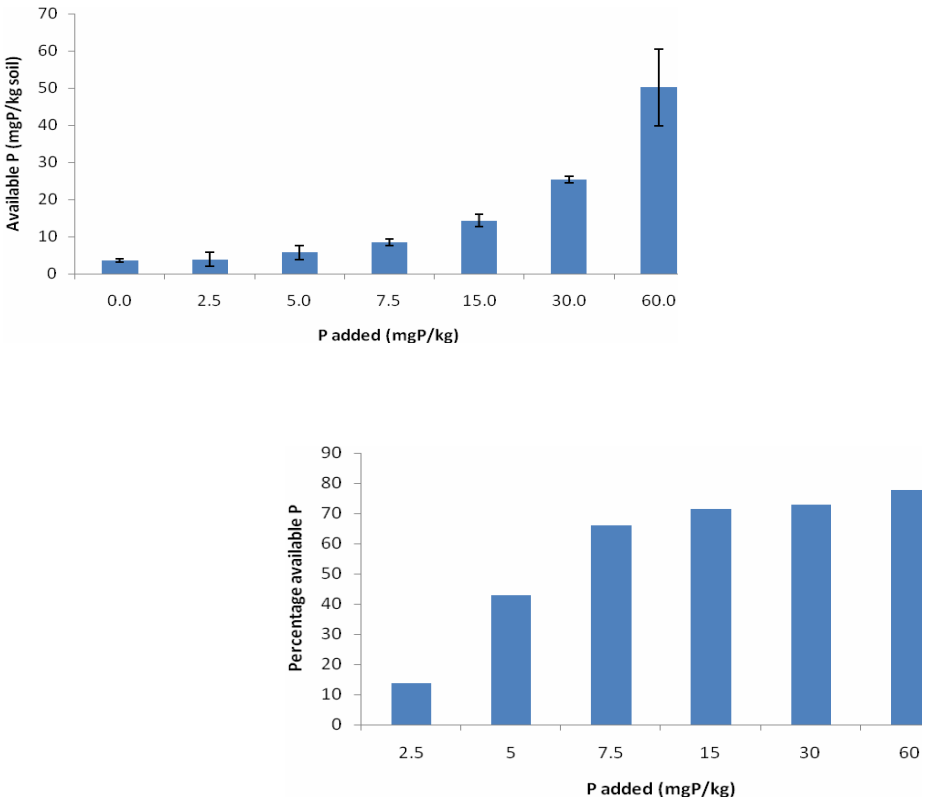

Figure 1. Relationship between P addition and available P expressed as mg/kg (a) and percentage of added P (b).

Experiment 2
After 6 weeks, AM colonisation was highest (20% colonisation) in soil with no P addition (Figure 2). Inorganic P addition significantly decreased the percentage of colonisation compared with the control soil without added P. The addition of ≥ 12.5 mgP/kg, decreased the colonisation to less than a half of the control; only around 5-7%.

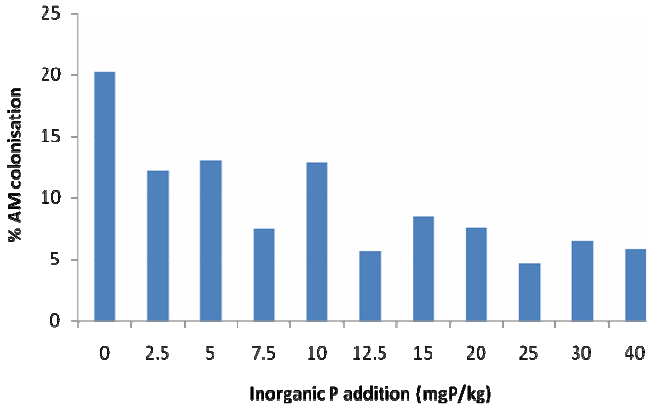

Figure 2. Percentage of AM colonisation of wheat in different rate inorganic P.

Experiment 3

The total P concentration in the residues was 0.67171, 2.08904 mg/kg in chickpea (CP), 6.553 mg/kg in faba bean mature shoot (FMS), 6.553 mg/kg in faba bean young shoot (FYS) and 8.31976 mg/kg in faba bean young root. For chickpea and faba bean mature shoot available P was very low and not significantly different between the rates of residue addition (Figure 3) . For faba bean young shoot and roots, available P increased with increasing residue addition rate with the greatest increase between 0.5 and 1% addition rate. As expected, available P nearly doubled. The increase between 1 and 1.5 % and 1.5 to 2% was smaller and did not match the increased amount of P added with the residues. This could be due to a smaller percentage of residues being decomposed at higher rates of residue addition.

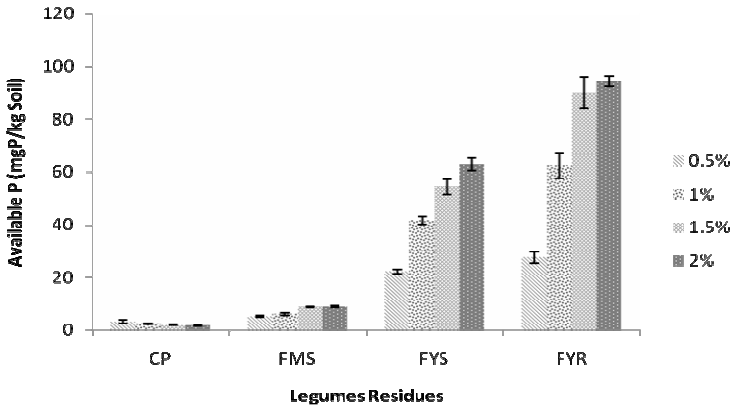

Figure 3. Available P in four different residue types and rates.

Conclusion

From these experiment, it is clear that the addition inorganic P lowered the AM colonisation particularly at more than 10 mg/kg available P. Hence, both chickpea and faba bean mature shoots will be used in the main experiment as sources of low available P because addition of these residues resulted in available P ≤10mgP/kg soil. On the other hand, faba bean young shoots and roots which had high total P concentrations resulted in > 20 mgP/kg soil available P even at 0.5% addition rate. Based on the increase in available P, these two residues may have a negative effect to AM colonisation. However, these residues may have a different effect if compared with inorganic P addition, e.g. addition of organic matter and/or stimulation of microbial activity via the residues may also stimulate AM colonisation despite the high concentrations of available P.

The study will provide important information about the link between legumes, AM colonisation and P uptake by wheat in an Australian soil. The finding of this research will contribute to the development of farming systems with increased growth and yield of wheat and less reliance on inorganic P fertilisers.

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Enzymatic activities in the rhizosphere of different plants at a glacier forefield

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Abstract
Availabilities of nitrogen (N) and phosphorus (P) are important determinants of primary ecosystem succession. One of the plant strategies to acquire sufficient amounts of P and N in young ecosystems like glacier forefields is to establish mycorrhizal symbiosis. Mycorrhizal fungi have developed different mechanisms to acquire nutrients from soils such as extensive mycelium growth, exudation of organic acids and/or lytic enzymes. Large amounts of such enzymes are produced by many ericoid (ER) and some ectomycorrhizal (ECM) fungi, thus providing access to organic forms of the nutrients. In contrast, arbuscular mycorrhizas (AM) are rather functioning as efficient pumps of soluble N and P forms from the soil solution, with limited access to the recalcitrant forms of nutrients. To quantify differences in enzymatic activities between ER, ECM and AM types, rhizosphere soils from four different plants species were collected at different soil developmental stages in a glacier forefield in Switzerland. Activities of different enzymes in the soil samples were assessed using fluorogenic substrates. Elevated chitinase and protease activities were associated with ECM and ER types, respectively, whereas no clear trends were observed for acid phosphatase. Soil developmental stage was an important factor of the background enzymatic activity levels.

Key Words
Mycorrhiza, nitrogen cycle, phosphorus cycle, soil enzymes, soil formation gradient.

Introduction

Development of soils and patterns of ecosystem succession on glacier forefields have been studied over the last years from different points of view. Among them, issues such as availability, turnover and cycling of nitrogen (N) and phosphorus (P) received particular attention of botanists, mycologists and microbiologists. It has been shown that the size of N and P pools as well as their forms vary strongly with soil developmental stages, which has consequences for nutrient availabilities for plants and soil microorganisms. On areas that have only recently been deglaciated, inorganic forms of N and P predominate, which originate either from parent rock weathering and/or deposition. Concentrations of both N and P are usually very low in those young soils. In more developed soils, significant accumulation of N and P is frequently observed and those nutrients are usually present in organic polymers (such as DNA, proteins, chitin etc.). To cover their nutritional demands, plants colonizing glacier forefields employ various mechanisms to acquire N and P from the different soil pools. Among them, formation of mycorrhizal symbioses is of major importance. About 90% of all terrestrial vascular plant species form mycorrhizas (Brundrett 2002), of which the arbuscular mycorrhizas (AM), ectomycorrhizas (ECM) and ericoid mycorrhizas (ER) are the most common types (Smith and Read 2008). Mycorrhizas are known to enhance N and P acquisition of their host plants. However, the mechanisms behind these processes are not the same for all mycorrhizal types. For example, AM fungi can efficiently gather soluble inorganic N and P from the soil solution, whereas their direct access to recalcitrant nutrient sources is very limited. In contrast, some ECM and ER fungi were shown to exude large amounts of exoenzymes and organic acids, which facilitate N and P uptake from recalcitrant organic and inorganic pools such as proteins and apatites (Smith and Read 2008).
Enzymatic activities of different mycorrhizal fungi have been recently studied in pure cultures (Bajwa and Read 1986; Joner *et al.* 2000; Read and Perez-Moreno 2003; Jayakumar and Tan 2005), which may be of limited relevance to natural ecosystems. For that reason, the main aim of this study was to investigate enzymatic activity of rhizosphere samples taken directly from natural conditions. We expected to find different enzymatic activities in different mycorrhizal types formed by various plant species along a soil formation gradient. This study was embedded in the interdisciplinary BigLink project (Bernasconi *et al.* 2008).

Methods

Study area

This study was carried out at the forefield of the Damma glacier, situated in Urner Alps, Switzerland (N 46°38', E 08°27') (Figure 1A). In July 2009, eight experimental sites were selected for enzymatic activity

investigations. These sites have been deglaciated for different periods of time, ranging from 7 to 110 years (Figure 1B). Approximate ages of soil in each of the experimental sites were calculated based on historic glaciological records available from 1921 through the Swiss Glacier Monitoring Network.

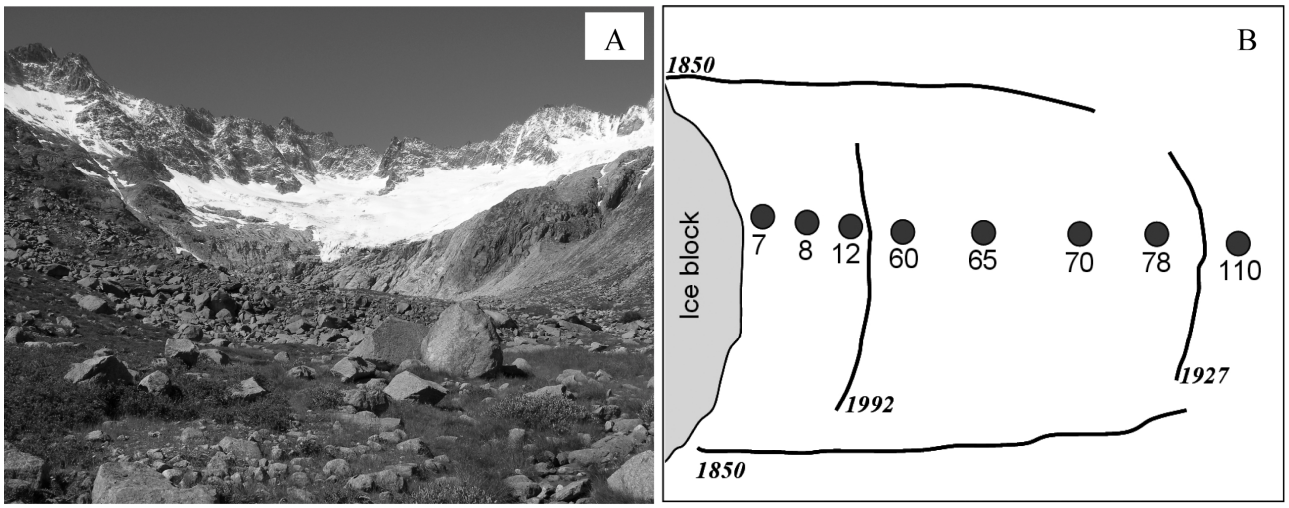

Figure 1. The Damma glacier forefield in the Swiss Alps (A), and schematic representation of the forefield (B). Points indicate individual experimental sites. Numbers below the points indicate approximate soil ages (times after deglaciation). Lines with dates indicate positions of side (1850) and end (1927 and 1992) moraines.

Soil sampling

Samples (~200 g each) were collected from each experimental site in the close vicinity of roots of four model plants, representing different mycorrhizal types: *Salix helvetica* (predominantly ECM plant), *Rhododendron ferrugineum* (ER plant), *Leucanthemopsis alpina* and *Agrostis gigantea* (both AM plants). From each site, an additional sample was collected from a spot not covered by vegetation. The soils were passed through a 2 mm sieve, aliquoted for subsamples of about 5 g each, and stored at -80°C. Soil carbon (C), N and P concentrations were assessed by standard methods (Table 1).

Table 1. Selected soil chemical properties

Site	Soil $pH_{(CaCl2)}$	Total C [A] (g/kg)	Total N [A] (g/kg)	Extractable P [B] (mg/kg)
1	4.37 (±0.36)	0.79 (±0.17)	0.03 (±0.03)	10.88 (±2.74)
2	4.49 (±0.28)	0.95 (±0.35)	0.05 (±0.05)	10.44 (±0.86)
3	4.17 (±0.07)	7.42 (±4.38)	0.44 (±0.16)	28.60 (±8.72)
4	4.13 (±0.09)	4.07 (±3.80)	0.22 (±0.20)	21.33 (±4.58)
5	4.10 (±0.07)	5.78 (±2.91)	0.36 (±0.17)	26.28 (±2.59)
6	4.03 (±0.18)	3.63 (±1.53)	0.22 (±0.11)	21.91 (±6.37)
7	3.91 (±0.16)	8.53 (±3.04)	0.42 (±0.17)	13.55 (±5.30)
8	3.72 (±0.16)	17.49 (±10.96)	0.95 (±0.63)	15.87 (±3.25)

Values are means of five analytical replicates (± standard deviation), [A] Dry combustion method, [B] Ammonium acetate-EDTA extraction followed by colorimetric quantification with malachite green as a color agent.

Enzyme assays and measurements

Enzymatic activities were assessed in soil suspensions. Approximately 1 g of soil was suspended in 40 mL of sterile distilled water, sonicated for 2 min (35 kHz) and shaken horizontally (2.3 Hz) for 1.5 h at room temperature. The suspensions were allowed to sediment for 15 min, after which the liquid samples were distributed into black 96-well microplates containing the relevant buffers. Acid phosphatase (EC 3.1.3.2) and chitinase (EC 3.2.1.14) assays were buffered with MES (Stemmer 2002), whereas the protease (leucine aminopeptidase, E 3.4.11.1) assay required modified universal buffer (Stemmer 2004). Standards and fluorogenic substrates used for the assays followed the description by Stemmer (2002). Enzymatic activities were quantified using a Biotek FLx800 microplate fluorometer (excitation at 360 nm and emission at 460 nm). Measurements were done at the following incubation times: 0, 30, 60, 90, 120, and 180 minutes.

Results

For all soils, enzymes activities were strongly affected by plant species that differ in their mycorrhizal status, but varied also greatly between the sites. High variability in soil properties (Table 1) was observed across the sites, with a general increase in C and N contents, and decreasing pH with soil age. Phosphatase activity increased with soil age, but no clear differences could be observed between the different plant species (data not shown). The highest chitinase activity was observed in a site deglaciated 12 years ago (Figure 2A). High activities were recorded in the rhizospheres of *S. helvetica* and *R. ferrugineum*, which were ECM and ER plant, respectively. Protease activity followed similar pattern to chitinase, but the values were several fold lower than those of chitinase (Figure 2B). Elevated protease activity (as compared to unvegetated soil) was sometimes associated with *S. helvetica* and *R. ferrugineum*.

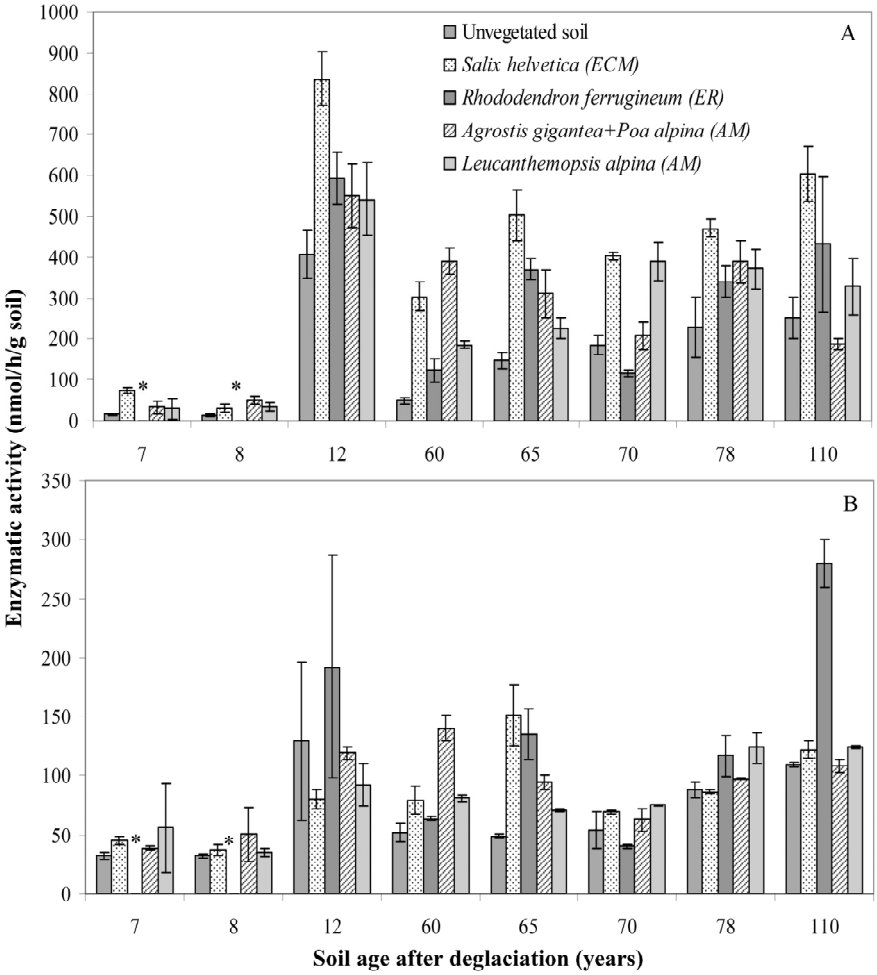

Figure 2. Chitinase (A) and protease (B) activities in rhizospheres of different plants growing in soils of different ages. Values are means of analytical replicates (n=3, ± standard deviation). Asterisks indicate absence of the plant species on a particular site.

Conclusion

The results of the present study showed that different mycorrhizal types, formed by various plant species along a soil formation gradient, differentially influenced enzymatic activities of their rhizospheres. While phosphatase is produced by many different organisms and this enzyme has its optimum pH at acidic conditions, increased acid phosphatase activity is intuitive along the soil formation gradient, where soil organic matter content dramatically increases with soil age (Table 1). High chitinase and protease activities were frequently associated with *Salix helvetica* and *Rhododendron ferrugineum*. This can be explained by the presence of ECM and ER mycorrhizal fungi associated with roots of these plants, with their capacity to exude proteases (Bajwa and Read 1986; Leake and Read 1991; Hodge *et al.* 1995). However, interpretation of enzymatic activities in the soil samples should also consider other ecosystem properties such as soil chemistry, plant cover composition and density, ecological optimum of the individual plant species as well as the levels of the mycorrhizal colonizations in their roots. These factors should receive appropriate attention in the future. Moreover, in order to better understand the origin and consequences of soil enzymatic

activities, further measurements of the enzymatic activities at the surface of roots are planned. Parallel measurements, scrutinizing enzymatic activities of the plant's roots collected in the field and cultivated in the growth chamber will be conducted. Plants inoculated or not with different mycorrhizal fungi will be compared for their root- and rhizosphere-bound enzymatic activities in the future, and quantification of fungal biomass by quantitative PCR is planned.

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Interactions between rhizosphere microorganisms and plants governing iron and phosphorus availability

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Abstract
Because Fe availability is low in most aerobic soil, microorganisms and plants release low molecular-weight compounds (chelators) which increase Fe availability. Microorganisms appear to be far more competitive than plants: they can utilise Fe bound to plant-derived chelators and decompose them, whereas microbial chelators are poor Fe sources for plants. However, some plants, such as grasses, grow well in Fe-deficient soils, which may be explained by the spatially and temporarily concentrated release of phytosiderophores. Plants and microorganisms have developed a number of strategies to increase soil P availability. Microorganisms can increase plant P uptake by mobilising more P than they require and by stimulating root growth and mycorrhizal colonisation. However, microorganisms may also decrease P availability by (i) net P immobilisation in their biomass, (ii) decomposition of P-mobilising root exudates and (iii) decreasing root growth or mycorrhizal colonisation.
Depending on the availability of carbon, the microbial biomass can influence Fe and P availability to plants by acting as either a source or a sink. We propose the following hypothesis: at high availability of carbon such as in the zone immediately behind the root tip, Fe and P immobilisation dominates, whereas in the mature root zones with decreased C availability, mineralisation is dominant. While net Fe and P immobilisation behind the root tip is likely to directly decrease plant uptake, net mineralisation along the mature root zones that have a low capacity for nutrient uptake may have a relatively small effect on plant uptake.

Key Words
Carbon availability, competition, microbial biomass, mobilisation, phytosiderophores, siderophores

Interactions between microorganisms and plants for iron

The total Fe content in soil is relatively high, but its availability to organisms is low in aerated soils because the prevalent form (Fe^{3+}) is poorly soluble. Plants and microorganisms have developed mechanisms to increase Fe uptake (Marschner 1995). In plants, there are two different strategies in response to Fe deficiency. Strategy I plants (dicots and non-graminaceous monocots) release organic acid anions which chelate Fe. Iron solubility is also increased by decreasing the rhizosphere pH, and Fe uptake is enhanced by an increased reducing capacity of the roots ($Fe^{3+} \rightarrow Fe^{2+}$). Strategy II plants (*Poaceae*) release phytosiderophores that chelate Fe^{3+}. Iron is taken up in the chelated form as Fe-phytosiderophore (Roemheld 1991; Von Wiren *et al.* 1993). Phytosiderophores are released only for a few hours per day at the root tip (Roemheld 1991).
Under Fe deficiency stress, microorganisms release organic acid anions or siderophores that chelate Fe^{3+}. After movement of the ferrated chelate to the cell surface, Fe^{3+} is reduced either outside or within the cell (Neilands 1984). Microorganisms produce a range of siderophores, e.g. ferrichromes by fungi, and enterobactin, pyoverdine and ferrioxamines by bacteria.
Bacterial siderophores are usually poor Fe sources for both monocot and dicot plants (Bar-Ness *et al.* 1992; Crowley *et al.* 1992; Walter *et al.* 1994). However, in some cases microbial siderophores have alleviated Fe deficiency-induced chlorosis in dicots (Jurkevitch *et al.* 1988; Sharma *et al.* 2003; Wang *et al.* 1993; Yehuda *et al.* 2000). On the other hand, plant-derived Fe-phytosiderophore complexes appear to be a good Fe source for bacteria (Jurkevitch *et al.* 1993; Marschner and Crowley 1998).
The interactions between different Fe chelators depend on the affinity of the chelators towards Fe and their relative concentrations. Compared to phytosiderophores, bacterial siderophores such as pyoverdine have a higher affinity towards Fe (Yehuda *et al.* 1996). If siderophores and phytosiderophores are present at similar concentrations, Fe is preferentially bound to the siderophores, which may even remove Fe from the Fe-phytosiderophore complex. In contrast to many bacterial siderophores, rhizoferrin from the fungus *Rhizopus arrhizus* has only a slightly higher affinity towards Fe compared to phytosiderophores. Rhizoferrin is a good

Fe source for barley, probably because of exchange of Fe from rhizoferrin to the phytosiderophore (Yehuda *et al.* 1996).
Thus, microorganisms appear to be highly competitive for Fe compared to plant roots. However, not only the affinity of the chelators towards Fe, but also their relative concentration is important (Yehuda *et al.* 1996). The diurnal rhythm of phytosiderophore release by grasses results in a high concentration of phytosiderophore at the root tips at certain times of the day (Crowley and Gries 1994). Under these conditions, phytosiderophores may be efficient Fe chelators that could even remove Fe from bacterial siderophores, particularly because the density of microorganisms at the root tip is low. Even if a proportion of the phytosiderophores is decomposed by microorganisms, the concentration remaining is likely to be sufficient to mobilise adequate amounts of Fe.

Interactions between microorganisms and plants for phosphorus
Although the total amount of P in the soil may be high, it is mainly present in forms that are unavailable to plants and microorganisms. Under P deficiency, plants may increase the soil volume exploited by increasing root growth and root hair length, or decreasing root diameter (Föhse and Jungk 1983). Plants and microorganisms can increase the solubility of inorganic P by releasing protons, OH^- or CO_2, and organic acid anions such as citrate, malate and oxalate; and they can mineralise organic P by release of various phosphatase enzymes. The effectiveness of these mechanisms may depend on soil type and/or P forms present in the soil. For example, a given citric acid concentration mobilized more P and had a more persistent effect in an oxisol than in a luvisol (Gerke 1992).
Rhizosphere microorganisms can increase or decrease the availability of P to plants (Marschner 2009). Rhizosphere microorganisms increase P uptake by solubilising or mineralising more P than they require and by stimulating root growth. They can also indirectly enhance plant P uptake by releasing plant growth regulators that stimulate root or root hair growth or mycorrhizal colonisation.
A large number of microorganisms with high P solublisation *in vitro* have been isolated (Banik and Dey 1983; Whitelaw *et al.* 1999). Inoculation with such P-solubilising microorganisms can either lead to increased P uptake and plant growth (Gerretsen, 1948; Kumar and Narula 1999; Kundu and Gaur 1980) or be ineffective (Azcon-Aguilar *et al.* 1986; Badr el-Din *et al.* 1986). Mycorrhizal fungi can also increase plant P uptake, but they will not be discussed in this overview.
Up to 80% of soil P can be in organic form (Richardson 2001). Phytate, which is considered to be the dominant form of organic P in soils (Turner *et al.* 2003), is a poor P source for some plants grown under sterile conditions (Hayes *et al.* 2000; Richardson *et al.* 2001). Microorganisms, on the other hand, excrete phytase which breaks down phytate (George *et al.* 2007; Richardson and Hadobas 1997); thus, inoculation with soil microorganisms strongly increased plant P uptake from phytate (Richardson *et al.* 2001).
Rhizosphere microorganisms can reduce plant P availability by immobilisation of P in the microbial biomass, decomposition of P-mobilising root exudates and by inhibition of root growth or mycorrhizal colonisation. Organic acid anions released by plant roots could potentially mobilise P, but are rapidly decomposed by soil microorganisms (Van Hees *et al.* 2002). However, roots may also release compounds such as phenolics or cell-wall degrading enzymes that inhibit microbial growth and hence decomposition of organic acid anions (Weisskopf *et al.* 2006).
In the presence of a readily available carbon source such as root exudates, P is rapidly immobilised in the microbial biomass. However, when C is depleted, microbial growth rates decrease and a proportion of the microbial biomass may die off, releasing P. Hence, an active microbial biomass with a high turnover rate can rapidly take up P, but may also represent a slow source of available P through release from dead microbial cells (Oberson *et al.* 2001; Seeling and Zasoski 1993). The importance of the microbial biomass for plant P uptake was shown to differ among plant families. Microbial biomass P in the rhizosphere was positively correlated with P uptake by three *Poaceae*, but not with P uptake by three Brassicas (Marschner *et al.* 2007), although the concentration of microbial biomass P in the rhizosphere of the species from two plant families was similar.

Hypothesis for the ratio of net nutrient immobilization and mobilization by the microbial biomass along the root axis
We hypothesise that the ratio of nutrient mobilisation to immobilisation and hence, plant Fe and P availability, varies along the root axis. At the root tip, where microbial density in the rhizosphere is relatively low, root exudates will be able to mobilise nutrients without strong competition by microorganisms. The high rate of root exudation at the root tip will stimulate growth of rhizosphere microorganisms immediately behind the root tip, accompanied by strong net Fe and P mobilisation. However, most of the mobilised Fe

and P will be taken up by the microorganisms; they may also take up Fe and P mobilised by root exudates; resulting in net immobilisation. A few centimeters behind the root tip, where root exudation is lower, Fe and P mobilisation will equal immobilisation and some of the P mobilised by root exudates can be taken up by the plant. In the older root zones, the lack of easily decomposable C sources results in lower microbial growth rates and hence Fe and P demand as well as death of microorganisms. Hence, microbial biomass Fe and P are likely to become available to the plant. However, plant uptake of the nutrients released from the microbial biomass in the older root zones may be low because of inherently low nutrient uptake rates in these root zones (Häussling *et al.* 1988; Colmer and Bloom 1998; Fang *et al.* 2007).

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Isolation and characterization of phosphate solubilizing bacteria from Chinese cabbage

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Abstract
Phosphate solubilizing bacteria (PSB) were isolated from the rhizosphere of Chinese cabbage and screened on the basis of their solubilization of inorganic tricalcium phosphate in liquid cultures. Ten strains that had higher solubilization potential were selected, and they also produced indole-3-acetic acid, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and siderophores. The strains were identified to be members of *Pseudomonas*, by 16S rDNA sequence analysis. Seed bacterization with PSB strains increased the root elongation and biomass of Chinese cabbage in seedling culture, although they had no effect on phosphorus uptake of plants. The plant growth promotion by PSB in this study could be due to the production of phytohormones or mechanisms other than phosphate solubilization, since they had no effect on P nutrition.

Key Words
Phosphate solubilizing bacteria, *Pseudomonas*, 1-aminocyclopropane-1-carboxylate, indole-3-acetic acid.

Introduction

Phosphorus (P), after nitrogen is the major plant growth-limiting nutrient despite being abundant in soils in both inorganic and organic forms. Chemical fertilizers added to the soils to circumvent the problem of P deficiency, further compound the situation by the fact that almost 75-90% of added P fertilizer is precipitated by Fe, Al and Ca complexes present in the soils (Gyaneshwar *et al*. 2002). Phosphorus biofertilizers in the form of micro-organisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Ryu, J.H *et al*. 2006). Individual or co-inoculation of PSB with other groups of microorganisms enhanced the plant growth by increasing the efficiency of biological nitrogen fixation or the availability of other trace elements and by the production of plant growth promoting (PGP) substances (Poonguzhali, S *et al*. 2005). To isolate and characterize the phosphate solubilizing bacteria (PSB) associated with the rhizosphere of Chinese cabbage (*Brassica campestris* sub sp. *pekinses*), using tri-calcium phosphate (TCP) as the insoluble phosphate source. Identification of the most promising PSB by 16S rDNA sequencing and studying their PGP characteristics like production of indolic compounds, 1-amino-1cyclopropance carboxylate (ACC) deaminase, siderophores and zinc (Zn) solubilization.

Methods

Collection of plant samples and isolation of phosphate solubilizing bacteria
Chinese cabbage plants sampled from the research plots of the experimental field at Cheongwon, Chungbuk, Republic of Korea, were immediately transferred to the laboratory and processed within 24h. The PSB from the rhizosphere and root interior were isolated on Pikovskaya agar medium with 0.5% TCP as the inorganic phosphate source using dilution and plating method.

Determination of P solubilization in plate and broth assays
Sixteen PSB strains were checked for solubilization halos in Pikovskaya and NBRIP media with 0.5% TCP as the phosphate source. The soluble P present in the culture supernatant of 48 h cultures grown with TCP was estimated by Murphey and Riley (1962) method.

Characterization of selected PSB by substrate utilization using BIOLOG plates
Pure cultures grown on Biolog Universal Growth agar for 24h and suspended in sterile saline (0.85% NaCl) were inoculated to the BIOLOG plates at 150 µl per well and incubated at 28°C for 48h. The plates were read with the multi-well plate reader at 595nm. Cluster analysis and dendogram was constructed using the UPGMA software.

Determination of plant growth-promoting characteristics of PSB isolates
Zinc solubilization was determined on plates supplemented with 0.1% Zn in the form of Zinc oxide. Presence of indolic compounds was determined spectrophotometrically at 530 nm using indole-3-acetic acid (IAA) as a standard with Salkowski reagent. Presence of ACC deaminase was checked in plates containing

DF salts minimal media supplemented with 3 mM ACC as the nitrogen source. Siderophores presence was checked using Chrome azurol-S (CAS) assay.

Identification of the selected PSB by 16S rDNA sequencing
The strains were identified by the analysis of their 16S rRNA gene sequences and sequence homologies were determined using BLAST. The identified gene sequences were submitted to GenBank/NCBI under the mentioned accession numbers

Results
Plant growth promotion by PSB included mechanisms other than solubilization of insoluble phosphates. Concurrent to this, the selected PSB strains from Chinese cabbage also efficiently solubilized insoluble ZnO and produced IAA. Except for strains CPBE30, CPBE43, and CPBE44, other strains produced siderophores. ACC deaminase activity of the strains ranged from 33.45 to 129.49 nmol of α-ketobutyrate released per min per mg protein (Table 1). A recent study showed that the endophytic *Pseudomonas rhodesiae* from red pepper promoted plant growth and induced systemic resistance of plants against *Xanthomonas*. The PSB strains in this study produced ACC deaminase, which stimulates plant root elongation through lowering the ethylene concentration in plants. The PREP activity (calculated as the percent increase of root length on bacterial inoculation over the uninoculated control) of the strains ranged from 10.30 to 53.0%, and the strain CPBE43 possessed the highest values for PREP and ACC deaminase activity. All the strains increased the root length of Chinese cabbage when compared with uninoculated control, although the values remained significant only when the PREP activity was greater than 50%. However, ACC deaminase activity alone could not be responsible for the PREP activity, since the isolate CPBR7 with higher ACC deaminase activity exhibited the least root length and similar results were also observed with a few other strains (Table 1).

Table 1. Solubilization of insoluble $Ca_3(PO_4)_2$ in plate and broth assays and other plant-growth promoting characteristics of selected bacterial isolates.

PSB isolate	P solubilization[a]			Zn[b]	IAA (μg/ml)[c]	Siderophore	ACC deaminase[*]	Root elongation[**]
	Solubilization index (%)	P (μg/ml)	pH					
CPBR6	183.3±28.9	326.0±2.90	5.17	1.37±0.05	1.95±0.68	+	77.2±7.04	5.22±0.4ba
CPBR7	155.6±9.6	305.1±11.0	5.24	1.33±0.06	1.88±0.04	+	108.3±7.70	4.45±0.2b
CPBR16	150.0±50.0	324.1±6.42	4.88	1.30±0.00	1.85±0.20	+	56.8±3.92	4.73±0.4ba
CPBE30	233.3±28.9	326.4±2.07	5.12	1.30±0.10	1.85±0.13	-	61.4±3.67	5.50±0.4ba
CPBE31	433.3±115.5	247.9±3.52	5.50	1.07±0.21	1.85±0.49	+	47.3±4.22	5.09±0ba
CPBE37	233.3±28.3	301.3±1.45	5.14	1.27±0.06	4.15±3.91	+	84.3±7.12	5.35±1.1ba
CPBE40	111.1±19.3	276.9±3.11	5.14	1.37±0.41	2.25±0.43	+	74.4±4.25	5.23±0.4ba
CPBE42	177.8±38.5	299.0±0.00	4.99	1.10±0.00	1.53±0.26	+	33.5±3.15	5.33±0.1ba
CPBE43	283.3±14.4	440.9±6.83	4.16	1.57±0.06	23.38±0.98	-	129.5±17.03	6.08±0.2a
CPBE44	163.3±4.72	282.1±0.83	5.44	1.23±0.25	2.35±1.24	-	79.4±11.20	5.43±0.5ba

[a]The solubilization index, determined as the proportion of the solubilization halo to the colony diameter on NBRIP agar and soluble P present in the culture supernatant at 48 h of growth in Pikovskaya broth with the corresponding reduction in pH.
[b]The diameter of the halo zone formed on Bunt and Rovira medium supplemented with 0.1% Zn.
[c]The amount of IAA in the culture supernatants supplemented with 100 μg/ml $_L$-tryptophan in the growth media; + and - indicate the presence or absence of siderophores as determined by CAS assay.
[*]nmol *α*-ketobutyrate released/min/mg protein; Each value represents a mean±standard deviation (SD) of three replications.
[**]The root length is given in cm; the value corresponding to uninoculated control is 4.0 cm (the average data of two replications with 10 plates per replication and 12 seedlings per plate). Within each vertical column, values followed by the same letter are not statistically different according to Fisher's protected LSD (P<0.05).

The 16S rDNA sequencing identified the strains to be *Pseudomonas*, showing close proximity with *Pseudomonas poae* (99.8-99.9%) and *Pseudomonas trivalis* (99.4-99.6%) reported from the phyllosphere of grasses, except for the strain CBPE30 showing 100% sequence similarity with *Rhizobium radiobacter* (Figure. 1).

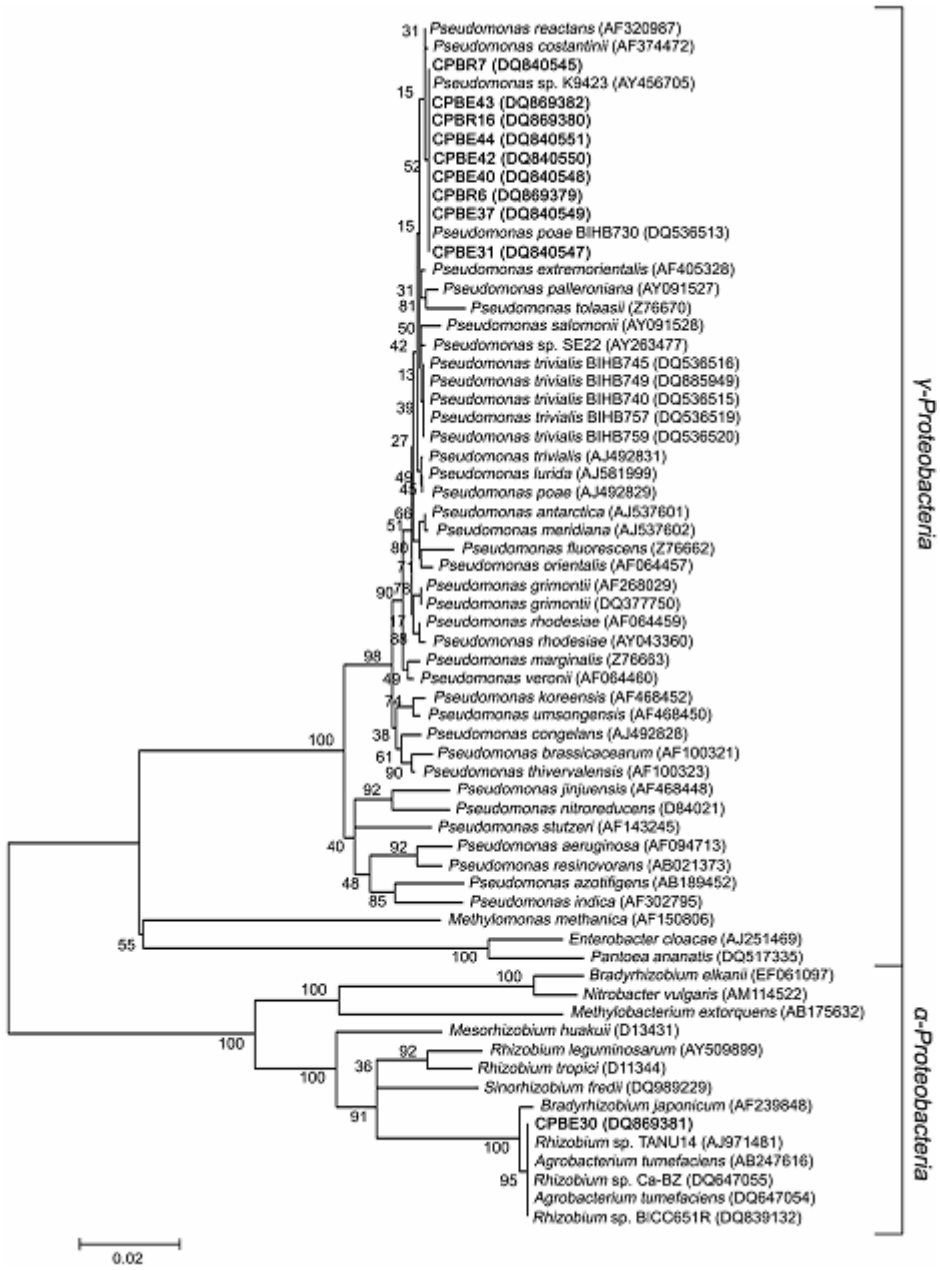

Figure 1. Phylogenetic tree based on 16S rDNA gene sequence comparison showing the position of the phosphate solubilizing bacterial strains isolated from Chinese cabbage and other related species of the genus.

The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bar represents0.02 substitutions per site. The strains of rhizosphere origin are designated as CPBR and those of endophytic origin as CPBE. The GenBank accession numbers are indicated in parentheses.

Application of PSB resulted in about 25% of reduction in P fertilizer, and increased the available P in soil and the sheath P status in sugarcane. However, inoculation of PSB strain had no effect on the P nutrition of plants, although the presence of metabolized root exudates by bacterial actions enhanced plant growth. Concurrent to this study, inoculation of bacterial strains to Chinese cabbage had no effect on the P concentration of plants. Soluble P in the extracts of inoculated plants remained less than that of the control, except for two strains, CPBE40 and CPBE42. However, bacterial inoculations through seed treatment increased the dry weight of plants, with an exception of strain CPBE44, and increased the available P in the external root solutions with an exception of strain CPBR6, in seedling cultures (Table 2). Hence, it is quite possible that the quantities of soluble P released from the insoluble phosphate source were too small or some

other sources may prevent their uptake by plants. Although acid phosphatases have nothing to do with the solubilization of inorganic phosphates, their synthesis is stimulated when the level of inorganic P in the growth medium is limited, thus making the apparent relationship between them co-incidental. The acid phosphomonoesterase activity of the root extracts showed higher values in bacterial inoculations, except for strains CPBE40 and CPBE43 that recorded lower values, 267.0 and 252.6 µg (PNP)/h/mg protein, respectively, than the control (Table 2). The present results revealed that the plant growth promotion by PSB strains from Chinese cabbage might possibly be due to the production of phytohormones or other mechanisms, since they had no effect on the P nutrition of plants. These indigenous PSB can potentially be exploited as PGPR for Chinese cabbage with further pot and field experiments because of its increased seeded acreage and commercial crop value in Korea.

Table 2. Soluble P content of root extracts and external root solutions, phosphomonoesterase activity of root extracts from PSB inoculated 19-day-old seedlings of Chinese cabbage.

Strain	Dry weight (mg plant^{-1})	Soluble P		Acid Phosphomono-esterase*
		Plant (µg g^{-1})	External solution (µg P ml^{-1})	
Pseudomonas poae CPBR6	18.0±1.73e	333.3±27.9e	0.56±0.06f	634.3±25.6a
Pseudomonas poae CPBR7	24.0±2.89d	495.1±31.8k	8.64±0.83b	430.9±17.9d
Pseudomonas poae CPBR16	76.0±4.62ba	435.0±26.0b	4.77±0.39d	455.7±32.2c
Rhizobium radiobacter CPBE30	73.0±3.46b	259.5±22.8f	9.95±1.07a	440.3±29.0dc
Pseudomonas trivalis CPBE31	30.0±2.31c	317.7±17.7e	5.87±0.56c	403.9±31.1e
Pseudomonas poae CPBE37	79.0±6.35a	269.6±14.8f	5.24±0.43dc	525.7±26.4b
Pseudomonas trivalis CPBE40	13.0±2.31fe	642.5±36.1a	8.55±0.89b	267.0±32.9g
Pseudomonas poae CPBE42	13.0±1.73fe	642.5±30.3a	1.83±0.08e	509.7±14.2b
Pseudomonas poae CPBE43	26.0±3.46dc	49.8±6.22h	6.03±0.31c	252.6±30.4g
Pseudomonas trivalis CPBE44	8.0±0.58f	161.8±10.3g	ND	403.6±19.4e
Uninoculated control	9.0±0.58f	536.0±21.9b	2.84±0.37e	358.9±28.2f
LSD (P≤0.05)	5.06	27.6	1.05	18.5

Values are the mean ± SE of three replicates. Within each vertical column, values followed by the same letter are not statistically different according to Fisher's protected LSD (P≤0.05). *µg (PNP) h^{-1} mg protein^{-1} enzyme activity of the root extracts.

Conclusion

The PSB isolates from Chinese cabbage apart from solubilization, also possessed other characteristics that may promote plant growth thus making them as a promising inoculant for crops. Further studies on the genetics of P solubilization and rhizospheric competence will help to develop them as successive bioinoculants.

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Isolation and evaluation of inoculation effect of *Azospirillum* sp. on growth, colonization and nutrient uptake of crops under green house condition

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Abstract
Nitrogen-fixing bacteria were isolated from the rhizosphere of different crops in Korea and were tested for their acetylene reduction activity (ARA). A total of 13 isolates were screened and were identified to belong to the reference strains of *Azospirillum.* The isolates were categorized into 2 groups- *A. brasilense and A. lipoferum* based on their sugar and biotin requirement. Among them *A. brasilense* was dominant and *A. brasilense* strains CW301 and CW903 showed the highest ARA. β-galactosidase activity verified through *pLA-lacZ* tagging confirmed their ability to colonize wheat root. Further investigation were made to verify the efficiency of *A. brasilense* CW903 to enhance crop growth and nutrient uptake of red pepper, tomato and rice under green house condition. Except for root lengths of red pepper and rice and root-shoot ratio of tomato, the inoculation of *A. brasilense* CW903 significantly improved other growth parameters ($P \leq 0.05$). Except for P, K, Mg and Zn in tomato, inoculation of *A. brasilense* CW903 appreciably enhanced the accumulation of other nutrients in rice and red peper. The consistent improvement in growth and nutrient uptake in non host crops suggest the potential of the *A. brasilense* CW903 for large scale field application.

Key Words
Plant growth-promoting rhizobacteria (PGPR), Nitrogen-fixing bacteria *, Azospirillum.* Acetylene reduction activity, β-galactosidase activity

Introduction
Azospirillum spp. isolated from various geographical regions of the world are the best-characterized genus of plant growth-promoting rhizobacteria (PGPR). They are known to associate with the roots of wheat, tropical grasses, maize, and other cereals (Okon and Hadar 1987; Oh *et al.* 1999). Members of the genus *Azospirillum* are gram-negative to gram-variable, curved-rod shape, motile, oxidase positive and exhibit acetylene-reduction activity (ARA) under micro-aerophilic conditions. *Azospirillum* spp. have been identified mainly as rhizosphere bacteria and its colonization of the rhizosphere has been studied extensively along with reporter gene fusion (Pereg-Gerk *et al.* 2000; Burdman *et al.* 1997; Holguin *et al.* 1999; Steenhoudt and Vanderleyden 2000). Recently, co-inoculation of *Azospirillum* sp. with *Methylobacterium* enhanced nutrient uptake by different crops as reported by Madhaiyan *et al*. (2009). The aims of this study were to isolate and identify efficient nitrogen fixing strains of *Azospirillum* spp. from the roots of various plants from Chungbuk province, South Korea, and to study their ability to colonize through *lacZ* fusions. Inoculation potential of strain *A. brasilense* CW903 on growth and nutrient uptake of rice, red pepper and tomato was also evaluated.

Methods
Isolation and characterization of N-fixing isolates
Screening of N-fixing organisms from the rhizosphere of different crops was carried out by the enrichment culture technique using semisolid malate medium (NFB) and further characterized by gram staining, glucose assimilation and biotin requirement (Baldani and Dobereiner 1980; Tarrand *et al.* 1978).

Acetylene-reduction assay
The acetylene-reduction assay (ARA) was performed on free-living cultures as well as on cultures in association with wheat plants. Ethylene formation was measured using a Varian model 3700 Gas chromatograph. The protein concentration was determined by Lowry method.

Sequencing of bacterial 16s rDNA and conjugation for reporter gene
Amplification for 16S rDNA was performed according to Devereux and Willis (1995) using primer 27F (5′-AGAGTTTGATCCTGG CTCAG-3′,), 1512R primer (5′- CGGCTACCTTGTTACGACT-3′). The amplified product was sequenced and compared with NCBI data base. On the basis of 16s rDNA comparison, five strains of *Azospirillum*, including reference strains, were conjugated with *Escherichia coli* S17.1 carrying the

plasmid *pLA-lacZ* to study colonization (Simon *et al*. 1983). Transconjugants were selected on minimal lactate medium supplemented with ammonium chloride (1 g/L) and tetracycline (5 µg/ml).

Effect of A. brasilense CW903 on plant colonization, plant-growth and nutrient uptake
In situ colonization study was verified in wheat seedlings according to Zeeman *et al*. (1992) in a growth chamber with a day-night cycle of 14 h (27 °C) and 10 h (18 °C) and β-galactosidase activity were determined 10 d after inoculation. Efficiency of *A. brasilense* CW903 on plant growth and nutrient uptake in red pepper, tomato and rice were evaluated under green house condition by seed bacterization. Bacterization of surface sterilized seeds was performed by imbibing the seeds in *A. brasilense* CW903 cell suspension (A600=0.5) for 6 h at 60 rpm. Seeds treated with sterile distilled water alone was considered as control. Seeds either inoculated with bacteria or untreated were sown in plastic pots (100 mm w x 75mm d x 85mm h) filled with approximately 250 g of air-dried wonjo mixed bed soil. The pots were held in racks (20 pots per rack) and grown under green house condition and watered regularly. Growth parameters such as shoot length, root length and root-shoot ratio were recorded 45 days after planting.
Total nitrogen was determined by a Kjeldahl Autoanalyzer Model 1030. Macronutrients (Ca, K, P and Mg) and micronutrients (Zn, Mn and Fe) absorbed by the plants were analysed using ICP-OES after acid digestion. Data were subjected to mean standard error (SE) and inoculation effect of *Azospirillum* on growth and nutrient uptakes were subjected to paired comparison.

Results

A total of 13 strains isolated from the rhizosphere of different plants grown in different regions of Korea were able to fix nitrogen (Table 1). These strains were isolated from a variety of plants including rice, wheat, Sudan grass, onions, and several dicotyledonous plants indicating that these nitrogen-fixing

Table 1. Characteristics of the N2-fixing strains isolated from the rhizosphere of different field-grown plants

Strains	Host plant	Gram reaction	Oxidase reaction	Potato infusion	C and N utilization		Group [a]
					Malic acid	Biotin	
Azospirillum brasilense Sp7[a]	-	-	+	Pink	+	-	I
Azospirillum brasilense CW5	Tobacco	-	+	Pink	+	-	I
Azospirillum brasilense CW202	Rice	-	+	Pink	+	-	I
Azospirillum brasilense CW301	Wheat	-	+	Pink	+	-	I
Azospirillum brasilense CW307	Wheat	-	+	Pink	+	-	I
Azospirillum brasilense CW406	Soybean	-	+	Pink	+	-	I
Azospirillum brasilense CW705	Rice	-	+	Pink	+	-	I
Azospirillum brasilense CW716	Onion	-	+	Pink	+	-	I
Azospirillum brasilense CW805	Onion	-	+	Pink	+	-	I
Azospirillum brasilense CW903	Taro	-	+	Pink	+	-	I
Azospirillum brasilense CW1401	Soybean	-	+	Pink	+	-	I
Azospirillum brasilense CW1402	Rice	-	+	Pink	+	-	I
Azospirillum brasilense CW1502	Apple	-	+	Pink	+	-	I
Azospirillum brasilense 687[b]	-	-	+	Pink	+	+	II
Azospirillum brasilense CW1503	Sudan grass	-	+	Pink	+	+	II

[a]The groupings are based on the similarities to the reference strains [a]*A. brasilens*e Sp7 and [b]*A. lipoferum* 687.

bacteria are inhabitants of the rhizosphere of many plant species. All the isolated strains were gram-negative, oxidase positive, Twelve strains showed characteristics of *A. brasilense* Sp7 and one strain (CW1503) showed characteristics of *A. lipoferum* 687. Among them, *A. brasilense* was found to be the dominant species on plant roots. There was wide variation in nitrogenase activity among the different isolates (Figure 1). *A. brasilense* CW301 and *A brasilense* CW903, which are associated with wheat roots and taro, had the highest ARA activity. The strains that showed the highest nitrogenase activity in wheat roots viz., *A. brasilense* CW301, *A. brasilense* CW903, *A. lipoferum* CW1503 were selected and their identifications were confirmed by 16S rDNA sequencing. 16 srDNA squencing confirmed 98% to 99% homology with *A. brasilense, A. lipoferum*, respectively, and were assigned the GenBank accession numbers AY518780, AY518777, AY518779, respectively. Further colonization studies with *A. brasilense* CW301, *A. brasilense* CW903 and *A. lipoferum* CW1503 showed considerable β-galactosidase activity under aerobic growth. Transconjugant of *A. brasilense* CW301 exhibited the highest activity followed by *A. lipoferum* CW1503 (data not shown).

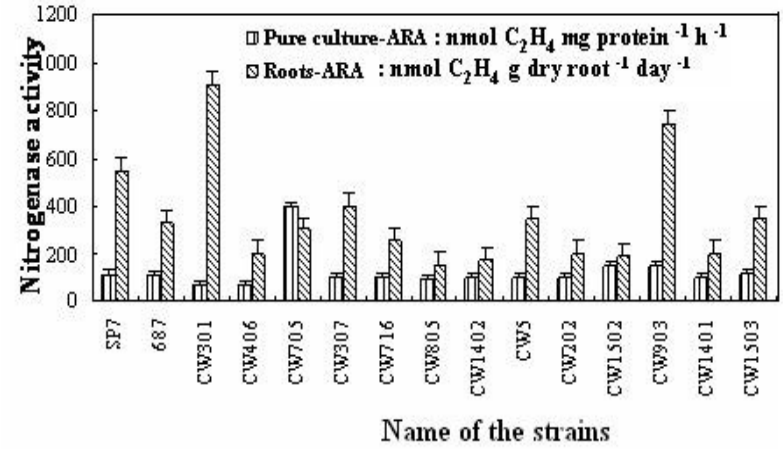

Figure 1. Nitrogenase activity of the isolates in semi-solid nitrogen free (NFB) medium and in association with the roots of wheat seedlings. Reference strains *Azospirillum brasilense* SP7 and *A. lipoferum* 687. Error bars represent standard deviation of observed values.

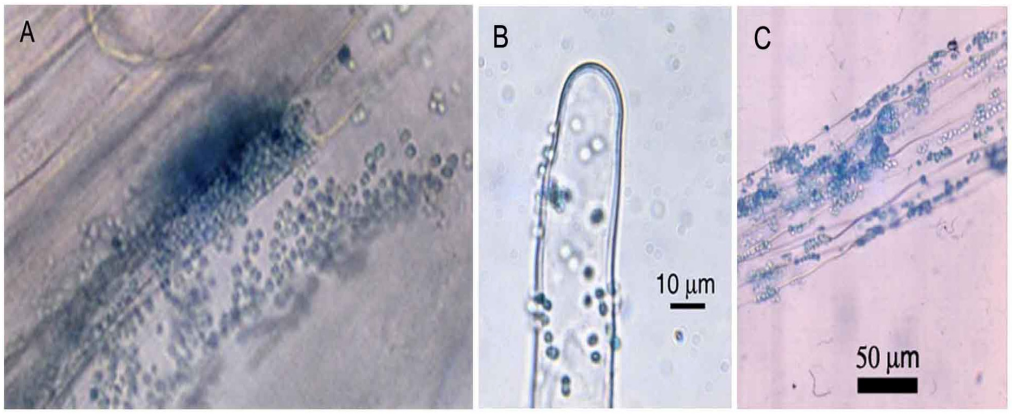

Figure 2. In situ detection of β-galactosidase activity on root segments of wheat plants grown after treatments of *Azospirillum brasilense* CW903. All samples were stained after 10 days of inoculation and X-gal staining was performed on five or six 1 cm root segments excised from the same plant starting from the tip. (A) root inoculated with *A. brasilense* CW903 (X60), (B) Root inoculated with *A. brasilense* (X100); (C) Root inoculated with *A. brasilense* SP7 (X40)

Examination of segments of wheat roots stained with X-Gal readily enabled the visualization of Azospirillum cells bearing *pLA-lacZ* plasmids (Figure 2). This confirms the colonization and multiplication ability of the strains along the root surface of the inoculated plants.

Effect of Azospirillum brasilense CW903 on growth and nutrient uptake of red pepper, rice and tomato
Except for root lengths of red pepper and rice and root-shoot ratio of tomato, treatment of *A. brasilense* CW903 significantly improved the growth of red peper, tomato and rice ($P \leq 0.05$) (Table 2). There was 18.7 – 26% increase in shoot length of red pepper, tomato and rice and 5.7 – 10.8% in root length of tomato.

Table 2. Effect of *Azospirillum brasilense* CW903 on the shoot, root length and root to shoot ratio of three different crops

Name of the plant	Treatments	Length (cm)		Root/shoot ratio
		Shoot	Root	
Red pepper	Control	12.19	16.70	0.435
	CW903	15.36*	18.51	0.502**
Tomato	Control	62.68	24.56	0.15
	CW903	75.05**	27.20**	0.19
Rice	Control	23.78	12.59	0.27
	CW903	28.21*	13.31	0.41*

The data are mean of four replications; *significant differences according to paired comparison.

Except for P, K, Mg and Zn in tomato, inoculation of *A. brasilense* CW903 appreciably enhanced the accumulation of total nutrients (Table 3). The total increment of the uptake of macro-and micronutrients by red pepper, tomato and rice were 12.0-26.7% N, 11.0-60.7% Ca, 4.8 – 77.5% Mn and 19.0 – 119.6% Fe.

Table 3. Effect of treatments of *Azospirillum brasilense* CW903 on total macro- and micronutrient content of different crops.

Name of The plant	Treatments	Macronutrient (mg/g dry plant biomass)					Micronutrient (□g/g dry plant biomass)		
		N	P	K	Ca	Mg	Mn	Zn	Fe
Red pepper	Control	3.98	0.56	57.06	14.51	6.82	0.17	0.10	0.46
	CW903	5.04*	0.63	70.53*	17.64	9.06*	0.20	0.34*	1.01*
Tomato	Control	1.67	0.58	60.56	9.86	4.69	0.21	0.12	0.13
	CW903	1.87	0.53	48.26	10.94*	4.28	0.22	0.08	0.17
Rice	Control	1.67	0.49	23.57	2.31	2.70	0.40	0.11	0.79
	CW903	2.07*	0.56	32.48*	3.71*	3.12*	0.71*	0.25	0.94*

The data are mean of four replications; *significant differences according to paired comparison.

Conclusion

Out of the 13 isolates, the strains *A. brasilense* CW301 and CW 903 showed highest ARA activity indicating that these strains may be suitable for inoculating wheat and other crops. β-galactosidase activity confirms that the strains are able to colonize introduced plants. Further inoculation of *A. brasilense* CW 903 on three crop plants prove the potential of this strain to enhance crop growth and nutrient uptake under green house conditions. The consistent improvement in growth and nutrient uptake of red pepper and rice plants by *A. brasilense* CW903 suggest the potential of this strain for large scale field application.

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Isolation of *Atriplex nummularia*-associated halotolerant bacteria and bioprospecting by nitrogen fixing bacteria in saline-sodic soil

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Abstract
The plant-associated habitat is a dynamic environment exploited by a wide variety of bacteria. These bacteria can contribute to the health, growth and development of plants by different mechanisms. *Atriplex* plants cultivated in saline sodic soils stimulated a bioprospection of bacteria-*Atriplex* interaction mechanisms. Therefore, the aim of this work was to study the interaction *Atriplex nummularia*- halotolerant bacteria and bioprospect nitrogen fixing bacteria. Leaf and root endophytic bacteria and bacteria from rhizosphere, cultivated soil and uncultivated soil were isolated using 10% TSA plus 50 g l^{-1} of NaCl. Furthermore, the screening for nitrogen fixing bacteria was evaluated by the ability to grow in semi-solid nitrogen-free NFb medium. The numbers of total cultivable halotolerant bacteria were significant between leaves, roots and rhizoplane of *A. nummularia* plants. The NFb medium methodology revealed that 56% of analyzed halotolerant isolates were able to grow in nitrogen free medium. The percentage of nitrogen fixing bacteria was higher for the bacteria in close interaction with the host plant than for the less related niches. The results from this study indicated that *Atriplex nummularia*-associated halotolerant bacteria are able to fix nitrogen and were found at higher percentage in close interaction with the host plant.

Key Words
Salinity, endophytic bacteria, interaction bacteria-plant, plant growth promotion.

Introduction
Plants may be considered to be a complex microecosystem where different niches are exploited by a wide variety of bacteria. Such niches include not only the external surfaces of plants, but also the internal tissues into which endophytic bacteria inhabit the interior of plants showing no apparently harm to the host or external structures (Azevedo *et al.* 2002). The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue (Mocali *et al*. 2003; Kuklinsky-Sobral *et al.* 2004), plant species and cultivar, soil type (Fromin *et al*. 2001; Kuklinsky-Sobral *et al*. 2004) and interaction with other beneficial or pathogenic microorganisms (Araújo *et al*. 2002; Lacava *et al*. 2007). Soil and plant-associated bacteria can contribute to the health, growth and development of plants by different mechanisms, such nitrogen fixing or production of phytohormones (Rosenblueth and Martinez-Romero 2006). The *Atriplex* spp. are salt tolerant, producers of biomass with high content of crude protein and are used as forage fodder shrubs (Bilal *et al.* 1990). These plants cultivated in low-fertility saline sodic soils stimulated a bioprospection of bacteria-*Atriplex* interaction mechanisms. Therefore, the aim of this work was to study the interaction *Atriplex nummularia*- halotolerant bacteria and bioprospect nitrogen fixing bacteria.

Methods
Plant and experimental field design
A field experiment was made with *Atriplex nummularia* cultivated in a saline-sodic soil (CE = 42.56 dS m^{-1}; PST = 71.20%) at Pernambuco State, Brazil (8º 34' 17" South and 37º 1' 20" West) during one year in a randomized block design, without irrigation. The soil was cultivated with plants in two treatments (with and without cutting at six months) and an uncultivated soil treatment, in four replicates.

Isolation of soil and Atriplex nummularia associated bacteria
Atriplex nummularia plants, rhizosphere, cultivated soil and uncultivated soil (0–0.2m layer) samples were collected and immediately transported to the laboratory where the plants were washed in running tap water to remove soil and the leaves and roots were separated.

Bacteria from rhizosphere, cultivated soil and uncultivated soil were isolated by placing five grams of soil in a 500 ml erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 ml of phosphate buffered saline [PBS, containing (g l^{-1}) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl 8.00; pH 7.4] and agitating the flasks at 100 rpm, 28°C for 1 h. Rhizoplane bacteria were isolated by placing three grams of root tissue in a 500 ml erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 ml of PBS and agitating the flasks at 100 rpm, 28°C for 1 h. After agitation, appropriate dilutions of the contents of the flasks were plated onto 10% trypicase soy agar (TSA) plus 50 g l^{-1} of NaCl and supplemented with 50 μg ml^{-1} of the fungicide Thiophanate methyl (Cercobin 700 PM, DuPont) and the plates incubated at 28°C for 2 to 15 days, after which colonies were picked off the plates, inoculated on 10% TSA agar slants, incubated at 28°C for 2 days and stored at 4°C. These colonies also were cultivated in 10% TSA, incubated at 28°C for 18 hours and following each culture was suspended in 20% glycerol solution and stored at -20°C.

Leaf and root endophytic bacteria were isolated according to Kuklinsky-Sobral *et al.* (2004) with some modifications. The surface disinfection process was done using serial washing in 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl^-) for 3 min, 70% ethanol for 30s and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto 10% TSA supplemented with 50 μg ml^{-1} of the fungicide Thiophanate methyl and incubating the plates at 28°C for 2 to 15 days. After surface disinfection, the leaf and root tissue was cut and triturated in 10 ml of sterile PBS contained in a 50 ml flask maintained at 28°C and agitated at 150 rpm for 1 h, after which appropriate dilutions were plated onto 10% TSA plus 50 g l^{-1} of NaCl and supplemented with 50 μg ml^{-1} of the fungicide Thiophanate methyl and incubated at 28°C for 2 to 14 days. After incubation, colonies were picked off the plates, inoculated on 10% TSA agar slants, incubated at 28°C for 2 days and stored at 4°C. These colonies also were cultivated in 10% TSA, incubated at 28°C for 18 hours and following each culture was suspended in 20% glycerol solution and stored at -20°C.

Screening for nitrogen fixing bacteria
The screening for nitrogen fixing bacteria was evaluated by the ability to grow in semi-solid nitrogen-free NFb medium (Dobereiner *et al.* 1995); a halo of bacterial growth within the medium indicates nitrogen fixation.

Results and Discussion
The interaction between halotolerant bacteria and *Atriplex nummularia* plants was assessed in leaves, roots, rhizoplane, rhizosphere, cultivated soil and uncultivated soil. Several bacterial morphotypes were observed on 10% TSA medium plus NaCl 50g l^{-1} (Figure 1). The number of total cultivable halotolerant bacteria was not significantly different among the soil treatments and rhizosphere, but was significant between leaves, roots and rhizoplane of *A. nummularia* plants (Figure 2). The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue, plant species and cultivar, soil type (Fromin *et al.* 2001; Mocali *et al.* 2003; Kuklinsky-Sobral *et al.* 2004). An understanding of the structure and species composition of plant-associated bacterial populations is fundamental to understanding how plant-associated biological processes are influenced by environmental factors and, consequently, has important biotechnological implications.

A total of 41 *A. nummularia*-associated halotolerant bacteria isolated from leaves, roots, rhizoplane, rhizosphere, cultivated soil and uncultivated soil were randomly picked up and were evaluated for their possible ability to fix atmospheric nitrogen. The methodology used was bacterial growth in nitrogen free medium (NFb medium), a halo of bacterial growth within the medium revealed nitrogen fixation (Figure 3). The NFb medium methodology revealed that 56% of analyzed halotolerant isolates were able to grow in nitrogen free medium. In this context, Bilal *et al.* (1990) observed root-associated nitrogenase activity in *Atriplex* spp. However the percentage of nitrogen fixing bacteria was higher (more than 60%) for the bacteria in close interaction with the host plant than for the less related niches (Figure 4). As described previously (Elvira-Recuenco and van Vuurde 2000) the roots seem to be the preferential site for epiphytic and endophytic bacteria, suggesting that endophytic bacteria may travel upward from the roots into the stem during plant development.

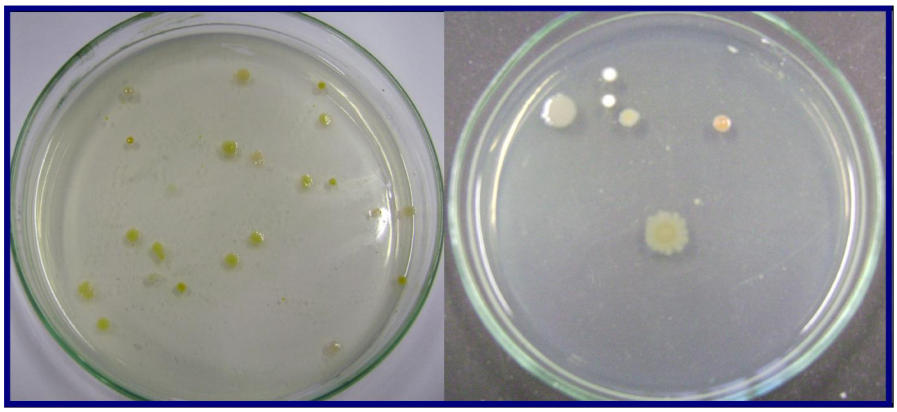

Figure 1. Morphotypes of *Atriplex nummularia*-associated halotolerant bacteria.

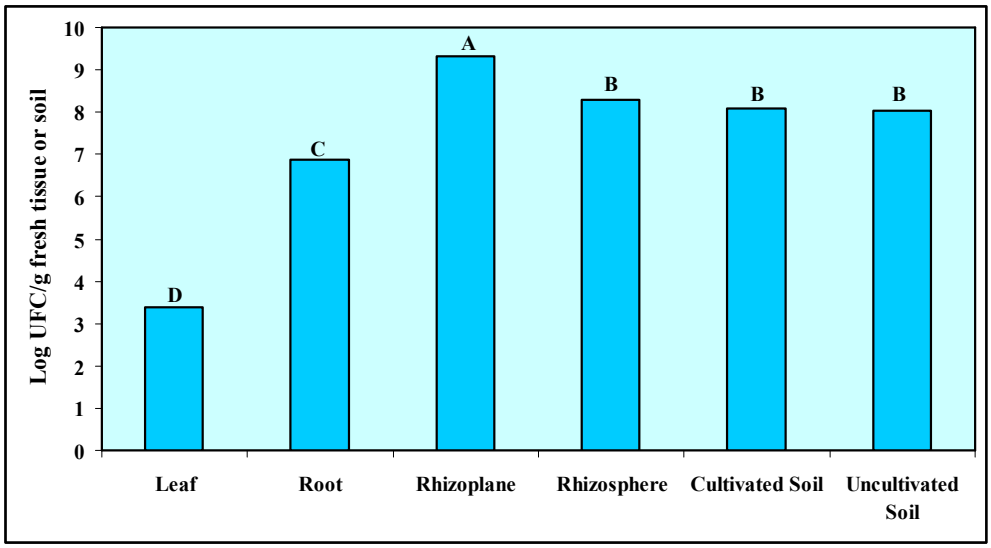

Figure 2. Total population density of *A. nummularia*-associated halotolerant bacteria isolated from: leaf, root, rhizoplane, rhizosphere, cultivated soil and uncultivated soil. Means with the different letters are significantly different by the Tukey test (P<0.05).

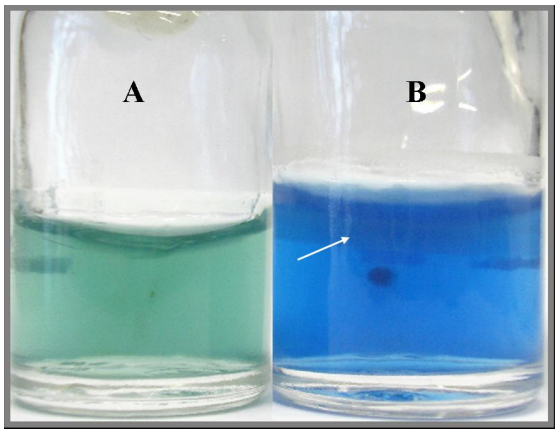

Figure 3. Negative (A) and positive (B) reaction to the test of biological nitrogen fixation in NFb medium. The arrow indicates the halo of bacterial growth.

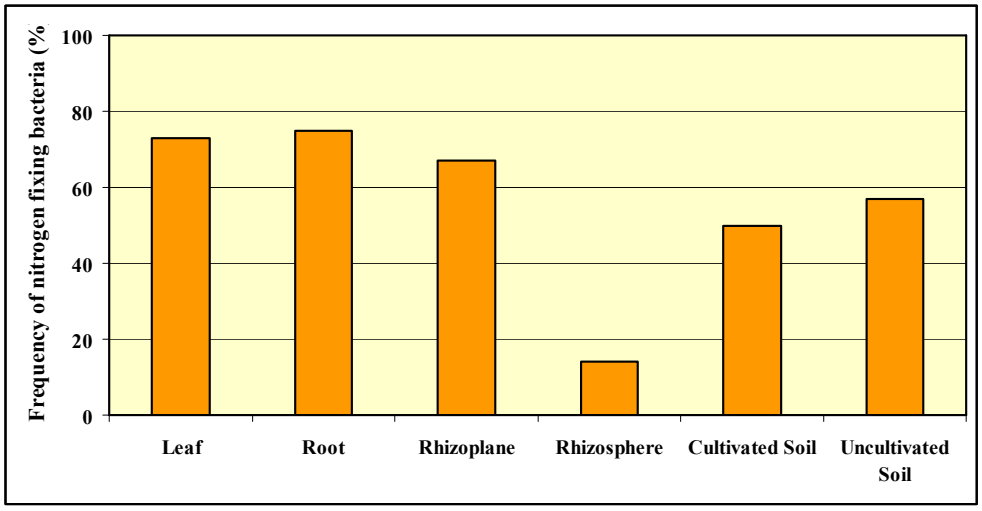

Figure 4. Frequency of nitrogen fixing *A. nummularia*-associated bacteria isolated from: leaf, root, rhizoplane, rhizosphere, cultivated soil and uncultivated soil.

Conclusion

The results from this study indicated that *Atriplex nummularia*-associated halotolerant bacteria are able to fix nitrogen and were found at higher percentage in close interaction with the host plant. However, a more complete comprehension of the interaction between *A. nummularia* and associated halotolerant bacterial communities is an important factor for a more effective crop management and should be further evaluated in field experiments.

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Microbial community composition under adjacent coniferous and broadleaf plantation forests

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Abstract
The PLFA profile technique was used to analyse soil microbial community composition under adjacent coniferous Chinese fir (*Cunninghamia lanceolata* (Lamb) Hook) (12-year old) (designated as 'Fir'), coniferous-broadleaf mixed species of broadleaf Nagai Podocarpus (*Nageia nagi* (thumb.) O. Ktze) and coniferous Pond cypress (*Taxodium ascendens* Brongn) (12-year old) (designated as 'Mixed Species') and broadleaf Phoebe Nanmu (*Phoebe Bournei* (Hemsl.) Yang) (12-year old) (designated as 'Broadleaf Phoebe'). The phospholipid fatty acid (PLFA) profiling analysis showed that amounts of total bacteria in the soils under the Mixed Species were higher than under the Fir and Broadleaf Phoebe, while amounts of fungi in the soils under the Broadleaf Phoebe and Mixed Species were lower than under the Fir. Results from the principal component analysis (PCA) of PLFA data showed that PC1 and PC2 explained 44.5% of the variations in the PLFA. Different forest types could be clearly separated along PCs 1 and 2, which were mainly attributed to 14 major PLFAs. The different organic inputs and availability of soil C substrates may be responsible for the discrimination of the soil microbial communities among different forest types.

Introduction

Microbial community plays a key role in soil carbon (C) and nutrient transformation (Chen and Xu 2008). A number of techniques, such as community level physiological profiling (CLPP), phospholipids fatty acid (PLFA) analysis and PCR-based molecular methods, have been used for detecting shifts in microbial community composition as affected by land-use change, forest species and management practices (e.g. Grayston and Prescott 2005; Ramsey *et al.* 2006). It has been suggested PLFA analysis can better differentiate the treatment effects on microbial community composition than CLPP or PCR-based methods (Ramsey *et al.* 2006). The PLFA profiles and microbial communities in forest soils have been reported to be affected by liming, organic matter removal, soil compaction and moisture stress and tree species (e.g. Frostegård *et al.* 1993; Grayston and Prescott 2005). The objective of this study was to investigate effects of coniferous, broadleaf and mixed forest species on soil microbial community composition as revealed by the PLFA profiling technique.

Methods

Site description and sample collection

The research site was located within the Forest Research Station, Fujian Agriculture and Forest University in Nanping, Fujian Province (26°38′S, 117°57′E) in the subtropical area of southeastern China. Three adjacent plantation forests planted in 1996 were selected for this study. These included: a) coniferous Chinese fir (*Cunninghamia lanceolata* (Lamb) Hook) (12-year old) (designated as 'Fir'); b) coniferous-broadleaf mixed species of broadleaf Nagai Podocarpus (*Nageia nagi* (thumb.) O. Ktze) and coniferous Pond cypress (*Taxodium ascendens* Brongn) (12-year old) (designated as 'Mixed Species'); and c) broadleaf Phoebe Nanmu (*Phoebe Bournei* (Hemsl.) Yang) (12-year old) (designated as 'Broadleaf Phoebe'). The experimental site measured 0.3 ha in area (ca. 0.1 ha for each of forest types) on the slope facing the sun. The soil was a Typic Alliti-Udic Ferrosols (Soil Survey Staff 1999), with the parent material being slope and residual deposits weathering from gneiss. Three replicate sampling plots (10 x 15 m^2) of each of the three plantation forests were established in the positions of upper, middle and lower slopes. Fifteen soil cores were randomly collected from each plot at the 0-20 cm and 20-40 cm, using a 7.5 cm diameter auger and bulked.

Analysis of phospholipid fatty acid in soil

Soil phospholipid fatty acid (PLFA) was extracted and measured using the modified methods described by Frostegard (1993) and Kourtev (2002). The amounts of individual PLFAs were expressed as mole % of total PLFA.

Identification of microbial community in soil

The composition of soil microbial community was identified by microbial analysis software (Sherlock MIS 4.5 System, MIDI, USA) based on the spectrogram of specific PLFA. The amount of bacteria in soils was estimated from the sum of percentages of the following PLFAs: i15:0, a15:0, 15:0 3OH, i16:0, a16:0, 16:1 2OH, 16:1ω5c, 10Me 16:0, 16:1ω7c, i17:0, a17:0, 10Me 17:0, 18:1ω7c, cy19:0ω8c (Frostegård *et al.* 1993; Frostegård and Bååth 1996). The PLFAs i15:0, a15:0, i16:0, i17:0, a17:0, 10Me 16:0, 10Me 17:0 represent Gram positive bacteria, while 16:1ω5c, 16:1ω7c, 18:1ω7c, cy19:0ω8c represent Gram negative bacteria (Frostegård *et al.* 1993; Frostegård and Bååth 1996). The sum of percentages of 18:2ω6,9, 18:1ω9c and 18:3ω6c (6, 9, 12) was considered to represent the percentage of fungi (Frostegård and Bååth 1996; Karliński *et al.* 2007). The percentages of actinomycetes was estimated from the percentage of 10Me 18:0 (Frostegård and Bååth 1996).

Statistical analysis

PLFA profiling data were also conducted in SAS Version 9.1.3 for Windows. Data (mole %) on the PLFA profiles were log-transformed [log (n+1)] and were subject to principal component analysis (PCA) using Statistica Version 6.1 (Statsoft, Inc.).

Results

Across the three plantation forests, the PLFAs 16:0, 18:1ω9c, cy19:0ω8c, 18:2ω6,9c, 18:0, i16:0 and i15:0 were predominant in both 0-20 cm and 20-40 cm layers, accounting for 61.8% to 71.9% of total PLFA. In particular, the PLFAs 16:0 and 18:1ω9c were the most abundant in all soils under the three plantation forests (Table 1). The PLFA profiles in the 0-20 cm and 20-40 cm layers varied greatly across the three plantation forests. Over 25 types of PLFA were detectable in the 0-20 cm soil under the Mixed Species, while only 20 types of PLFA were found in the corresponding depth under both Fir and Broadleaf Phoebe (Table 1). In the 20-40 layers, the number of soil PLFA in three plantation forests followed the order: Mixed Species (20)＞ Fir (18)＞Broadleaf Phoebe (16) (Table 1).

Table 1. PLFA profiles from soils under adjacent coniferous Chinese fir (Fir), coniferous-broadleaf mixed species of broadleaf Nagai Podocarpus and coniferous Pond cypress (Mixed Species), and broadleaf Phoebe Nanmu (Broadleaf Phoebe) plantation forests in subtropical China.

PLFA	0-20 cm (Mole %)			20-40 cm (Mole %)		
	Fir	Mixed species	Broadleaf Phoebe	Fir	Mixed species	Broadleaf Phoebe
12:0	0.3a	0.3a	0.0a	0.0a	0.0a	0.0a
14:0	1.7a	1.8a	1.2a	1.8a	1.3a	1.2a
i15:0	5.0a	4.9a	5.3a	4.7a	5.0a	5.7a
a15:0	3.2a	2.7a	3.0a	3.0b	3.4b	4.8a
15:0 3OH	0.0b	2.0a	0.0b	0.0b	2.1a	0.0b
16:0	19.3b	17.5b	22.9a	21.9a	18.3a	20.0a
i16:0	5.0a	4.6a	5.1a	4.3a	5.4a	4.8a
a16:0	0.0c	0.6b	1.1a	2.2a	0.0b	0.0b
16:1 2OH	0.0c	3.4a	2.7b	0.0b	3.9a	4.6a
16:1ω5c	2.3b	2.6b	3.5a	3.1a	3.8a	3.6a
10Me 16:0	3.7a	3.9a	2.9b	2.9a	3.5a	3.2a
16:1ω7c	1.6a	0.7a	1.4a	0.0a	0.6a	0.0a
i17:0	3.8a	3.3ab	2.7b	3.7a	4.0a	4.0a
a17:0	2.6a	2.3a	2.2a	2.5a	2.1a	3.3a
10Me 17:0	0.0b	0.8a	0.0b	0.0a	0.0a	0.0a
18:0	5.1a	4.2a	4.4a	5.7a	4.9a	6.1a
18:0 2OH	0.0a	0.0a	0.0a	1.9a	0.0a	2.6a
10Me 18:0	3.0a	3.2a	3.2a	0.0a	0.0a	0.0a
18:1ω7c	3.2a	3.4a	1.9b	3.1b	4.2a	0.0c
18:1ω9c	23.3a	17.8b	18.7b	24.7a	20.7b	22.2ab
18:2ω6,9c	5.0a	4.0a	5.0a	4.8a	4.9a	5.4a
18:3ω6c (6,9,12)	2.4a	2.4a	1.2b	3.6a	0.5b	0.8b
19:1ω6c	0.5b	2.3ab	3.4a	0.0a	1.1a	0.0a
cy19:0 ω 8c	7.9a	8.8a	8.2a	5.4b	9.4a	7.7ab
20:0	1.1ab	2.5a	0.0b	0.7a	0.9a	0.0a

Data in the row are mean values（n=3）, which are compared among forest types within each depth and are not different at the 5% level of significance if followed by the same letter.

This indicated that the soils under the mixed species of forest plantations contained more diverse PLFAs than those under mono-species forest plantations. In the 0-20 cm layer, amounts of PLFAs 16:0, a16:0, 16:1ω5c and 19:1ω6c under the Broadleaf Phoebe were significantly higher than those under the Fir and Mixed Species, while 15:0 3OH, 16:1 2OH, 10Me17:0 and 20:0 under the Mixed Species were remarkably higher than those under the Fir and Broadleaf Phoebe (Table 1). Amounts of the PLFAs 10Me16:0, i17:0, 18:1ω7c and 18:3ω6c (6,9,12) in the 0-20 cm soils were significantly higher under the Fir than those under the Broadleaf Phoebe, while those under the Mixed Species were intermediate. The PLFA 18:1ω9c in the 0-20 cm soils under the Fir was significantly higher than that under both Mixed Species and Broadleaf Phoebe (Table 1). In the 20-40 cm layer, the amounts of PLFAs a16:0, 18:1ω9c and 18:3ω6c (6,9,12) under the Fir were significantly higher than those under the Mixed Species and Broadleaf Phoebe, while amounts of 15:0 3OH, 18:1ω7c and cy19:0 ω 8c under the Mixed Species were higher than those under the Fir and Broadleaf Phoebe (Table 1). The amount of 16:1 2OH was significantly higher at both depths under the Mixed Species and Broadleaf Phoebe than those under the Fir (Table 1).

Results from the PCA have clearly showed the separation among the forest types and the soil depths along both PC1 and PC2 (Figure 1a). Three forest types, Fir, Mixed Species and Broadleaf Phoebe, had distinct PLFA compositions from each other. In addition, distinct PLFA patterns were also found between the 0-20 cm and 20-40 cm soils (Table 1, Figure 1a). The sum of PC1 and PC2 accounted for 44.5% of the variation in the PLFA composition. From the loading values of individual PLFA (Figure 1b), it is clear that the PLFAs 18:1ω9c, 18:1ω7c, 19:1ω6c, 18:00, a15:0, 10Me17:0, 10Me18:0 have made most important contributions to the separation of the 0-20 cm soils of different forest types (total contributions to the PC1: 55.8%), while 10Me16:0, cy19:0ω8c, i16:0, 16:1 2OH, a16:0 and 18:3ω6c (6, 9, 12) were most important in separating the 20-40 cm soils of different forest types (total contributions to the PC2: 65.5%). In addition, one of most abundant PLFAs, 16:0, contributed to 5% of variation in PC2, but 13% to the PC3 (data not shown).

Bacteria were the major microbial group in both 0-20 cm and 20-40 cm layers in the three plantation forests, accounting from 34% to 44% of total microbial community, followed by fungi with comprising 24%-34 %, and actinomycetes with up to 3.2%. Actinomycetes were only detected in the 0-20 cm layer (Table 2).

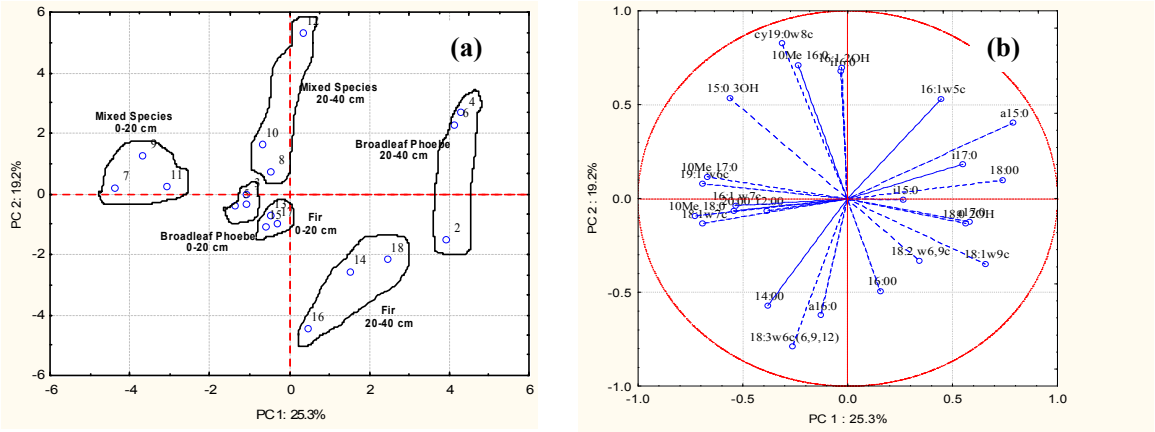

Figure 1. (a) Scores plot of PCA showing the separation of different forest types and soil depths along principal components (PC) 1 and 2; and (b) Loading values of the individual PLFA for PC1 and PC2.

Table 2. Mole percentages (%) of soil microbes under adjacent coniferous Chinese fir (Fir), coniferous-broadleaf mixed species of broadleaf Nagai Podocarpus and coniferous Pond cypress (Mixed species), and broadleaf Phoebe Nanmu (Broadleaf Phoebe) plantation forests in subtropical China.

Microbial community	0-20 cm			20-40 cm		
	Fir	Mixed species	Broadleaf Phoebe	Fir	Mixed species	Broadleaf Phoebe
Bacteria	38.3b	43.9a	40.0b	34.9b	46.3a	41.7ab
Gram positive bacteria	23.3a	22.5a	21.2a	21.1b	22.3b	25.8a
Gram negative bacteria	15.0a	15.4a	15.0a	11.6b	17.9a	11.3b
Fungus	30.7a	24.3b	24.9b	33.7a	26.2b	28.5b
Actinomycetes	3.0a	3.2a	3.2a	0.0a	0.0a	0.0a
Fungal-to-bacterial ratio	0.80a	0.57b	0.63b	0.97a	0.60b	0.67b

Data in the row are mean values（n=3）, which are compared among forest types within each depth and are not different at the 5% level of significance if followed by the same letter.

Amounts of total bacteria were higher in the soils at both depths under the Mixed Species than under the Fir and Broadleaf Phoebe, while there were no significant differences in the amount of bacteria under the Fir and Broadleaf Phoebe (Table 2). There were no significant differences in Gram positive and Gram negative bacteria in the soils under three plantation forests. On the other hand, amounts of fungi were higher in the soils under the Fir than under the Broadleaf Phoebe and Mixed Specie. The fungal-to-bacterial ratio was generally greater in the soils under the Fir than under the Broadleaf Phoebe and Mixed Species.

Conclusion

Different plantation forests had significant impacts on soil microbial community composition as revealed by the PLFA profiling. Amounts of total bacteria in the soils under the Mixed Species were higher than under the Fir and Broadleaf Phoebe, while amounts of fungi in the soils under the Broadleaf Phoebe and Mixed Species were lower than under the Fir. The PCA results showed that PC1 and PC2 explained 44.5% of variations in the PLFAs, and different forest types could be clearly separated along PCs 1 and 2, which were mainly attributed to 14 major PLFAs. The different organic inputs and availability of soil C substrates may be responsible for the discrimination of the soil microbial communities among different forest types.

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Microbial resilience as influenced by an herbicide in soils from native prairie, CRP, and row crop management

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Abstract
The objective of this study was to examine differences in soil microbial resilience as impacted by herbicide application on soils from prairie and row crop management. The treatments were: native prairie (TP), restored prairie (PF), CRP*, and corn-soybean rotation (CS) management. Surface soil (10 cm) was collected from two locations within each treatment with four subsamples per location in June 2008. Soil enzymes studied include: fluorescein diacetate (FDA) and dehydrogenase. Eleven glyphosate concentrations (0 to 2000 ppm) were applied (Roundup Original Max) and soil dehydrogenase and FDA hydrolase activities were assayed periodically over 50 days. Soil enzyme activities were lower on day one and ten irrespective of enzyme type or management. The highest enzyme activities were observed on day 5 and the activity declined thereafter. However, some soil treatments exhibited increased activity on days 30 and 50. Herbicide concentration had a smaller effect on FDA hydrolase activity while the effect of concentration was highly variable for dehydrogenase activity. Results of the study show that soils from native prairie have greater enzyme resilience while cropped soils have the lowest. Establishment of prairies may help maintain active soil processes and could help maintain or improve soil quality.

Key Words
corn-soybean, dehydrogenase, FDA, grassland ecosystems

Introduction

Herbicides are used in large quantities in modern agriculture to control undesirable plant species within a field and also to increase watershed or site productivity. The increased application of herbicides leads to increased chemical concentrations in soil, altered soil reactions, and potential adverse effects on non-target organisms (Perucci and Scarponi 1994). For example, 10- and 100-fold higher herbicide concentrations of imazethapyr reduced microbial biomass and activity of dehydrogenase enzyme in soils (Perucci and Scarponi 1994). In another study, respiration rates increased with higher concentrations of 2,4-D, picloram, and glyphosate (Wardle and Parkinson 1990). Ratcliff *et al*. (2006) observed 25% increase in bacterial counts and fungal hyphal length on glyphosate treated soils.

Modes of action for most herbicides are well defined. Glyphosate is a commonly used herbicide in many agricultural row crop management practices worldwide. Glyphosate inhibits protein synthesis by blocking the shikimic acid pathway (Franz *et al.* 1997). A surfactant, polyoxyethylene tallow amine, is toxic to bacteria and protozoa (Tsui and Chu 2003).

Biological and biochemically mediated processes in soils are significant for ecosystem functions (Zabaloy *et al.* 2008). Since microbes play a critical role in carbon (C) and nutrient transformations, any change in their population and activity may affect cycling of nutrients as well as availability of nutrients, thus indirectly affecting plant growth and other soil functions (Wang *et al.* 2008). Research suggests that repeated application of herbicides may involve a risk of reduced or altered soil microbial activities. Management practices may also indicate short-term differences in soil quality improvement and microbial diversity, soil chemical processes, mineralization rates, and organic matter accumulation. Other advantages of Research shows that fluorescein diacetate (FDA) hydrolysis and dehydrogenase activity can be used as good indicators of soil biogeochemical processes (Kremer and Li 2003). Enzyme assays among different enzyme assays include evaluation of rapid responses to changes in management and understanding sensitivity to environmental stresses (Dick 1997).

* CRP is a government incentive program in which previously cultivated land is taken out of crop production and maintained under a 15-yr continuous cool-season grass and legume pasture system with no agrichemical inputs.

Although several environmental benefits of restoration of prairies are reported in the literature, immediate quantifiable information on herbicide effects on these soils as compared to other soils is unavailable. This information should help explain changes in water and soil quality due to management practices, develop soil cleaning procedures, and assist in establishment of prairie vegetation establishment guidelines. The present study was designed to elucidate the effects of glyphosate (Roundup) on soil microbial resilience and potential soil enzyme activity in soils from native prairie, restored prairie, conservation reserve program (CRP), and crop management.

Materials and Methods

Soil dehydrogenase and FDA enzyme activities in response to varying concentrations of glyphosate and duration on soils from row crop and conservation reserve program (CRP*) treatments were compared to native prairie and restored prairie ecosystems in central Missouri, USA. The treatments for the study include the following: Tucker Prairie (TP; native prairie), Prairie Fork (PF; restored prairie), Conservation Reserve Program (CRP), and corn-soybean rotation (CS). The undisturbed Tucker Prairie site has been under native prairie vegetation and consists of big blue stem (*Andropogon gerardi* Vitman.), little blue stem (*Schizachyrium scoparium* Nash.), prairie dropseed (S*porobolus heterolepis* [A. Gray] A.Gray), and Indian grass (*Sorghastrum nutans* [L.J. Nash]) (Buyanovsky *et al.* 1987). The Prairie Fork Conservation Area was restored in 1993 with native grasses and legumes. Prior to restoration, the site was under row crop management for approximately 100 years. The study area vegetation consisted of little blue stem, side-oats gamma (*Bouteloua curtipendula var. curtipendula*), and Indian grass. Soils for the CRP and CS treatments were sampled from long-term study plots located within the USDA-ARS Agricultural Systems for Environmental Quality site near Centralia, Missouri, USA.

Five grams of soil was placed in a petri dish; moisture was maintained by adding water and keeping the lid in place in a control room at ambient temperature. Eleven glyphosate solutions (0 to 2000 ppm) were prepared by mixing the concentrated herbicide with DI water and approximately 1.5 mL of the solution was applied to the soils in the petri dish. The hydrolysis of fluorescein diacetate was colorimetrically quantified at 490 nm and indicates broad-spectrum soil enzyme activities, especially on substrates including carbohydrates, lipids, and proteins (Dick *et al.* 1996). A standard calibration curve was used to measure the concentration, expressed as μg fluorescein released g^{-1} dry soil h^{-1}. Dehydrogenase enzyme activity was determined in moist soil incubated with 2,3,5-triphenyltetrazolium chloride substrate at 37°C for 24 h (Pepper *et al.* 1995). Following incubation, a regression equation was used to determine the concentration of the triphenyl formazan (TPF) product colorimetrically (485 nm) and the enzymatic activity was expressed in μg TPF released g^{-1} dry soil h^{-1}.

Results

The dehydrogenase activity was significantly lower on day one for native prairie as compared to other three soils (Figure 1). The highest mean activity was observed in the CRP soil, which may have contained more soil carbon and may have received herbicides under previous cropping and from adjoining crop areas prior to this application; thus microbes have been less sensitive for additional herbicide application. The activity increased by almost three fold for restored prairie (PF) and CRP soils on day five. The native prairie site showed a fivefold increase from day one to day five. However, the crop soil had the lowest increment in change. The activity was the lowest on all four soils on day 10 and increased for days 30 and 50. This could possibly be due to the non-availability of organic carbon by day 10 and subsequent availability during days 30 and 50 due to increased carbon released from dying microbial biomass. A spike in microbes able to use less complex compounds derived from glyphosate after primary degradation by a more specialized, limited microbial community segment may also have contributed to the increase.

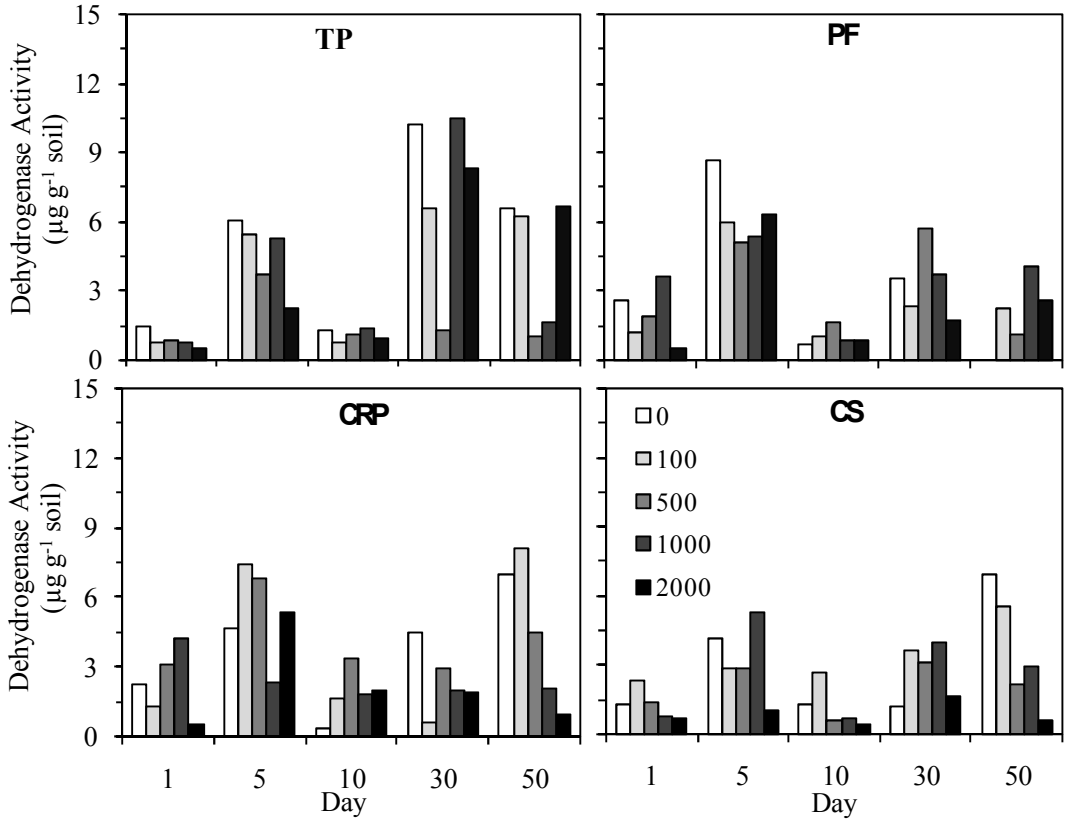

Figure 1. Dehydrogenase activity of native prairie (TP), restored prairie (PF), CRP, and corn-soybean rotation (CS) soils at 1, 5, 10, 30 and 50 days after application of 0 to 2000 PPM (selected concentrations are shown) concentrations of glyphosate.

Although the FDA enzyme activity was measured on all four soils and for several concentrations, results were presented for two selected soils (TP and CS) and five concentrations. Soils from the native prairie site exhibited the highest activity on days 1, 5, 10, 30, and 50 than the other three soils. This likely reflects the continuous presence of abundant and complex organic substances in the prairie ecosystem, which contributes to development of high diversity of enzymes required to degrade the complex organics. FDA activity of restored prairie and CRP were almost the same on day one. However, CRP soil had significantly greater activity on day five as compared to restored prairie soils. The differences were not significant between restored prairie and CRP soils for the rest of the study period. Soil from the corn-soybean rotation had the lowest activity among the four soils on all five measurement dates. The two prairie soils exhibited a 1.5 fold increase of enzyme activity from day one to day five while the CRP and CS soils exhibited two and five fold increases, respectively.

The effect of herbicide concentration on either enzyme activity within a measured date was insignificant by soil treatment. Although the highest concentration used in this study may represent an herbicide spill, results show that such high concentration did not affect enzyme activity. However, soil from the corn-soybean site had lower enzyme activity for higher herbicide concentrations. This might suggest that continuous soil disturbance and application of other agri-chemicals may have negatively affected microbial resilience for soils under crop management. Observed enzyme activities could be due to direct and/or indirect effects of pesticides which were not evaluated in this current study.

Conclusions

Results show that native prairie soils or soils with less disturbance had better enzyme resilience. This may indicate that establishment of permanent vegetation along the field crop borders may enhance microbial processes including degradation of harmful agri-chemicals, nutrient cycling, and carbon sequestration, and thus help protect soil and water quality.

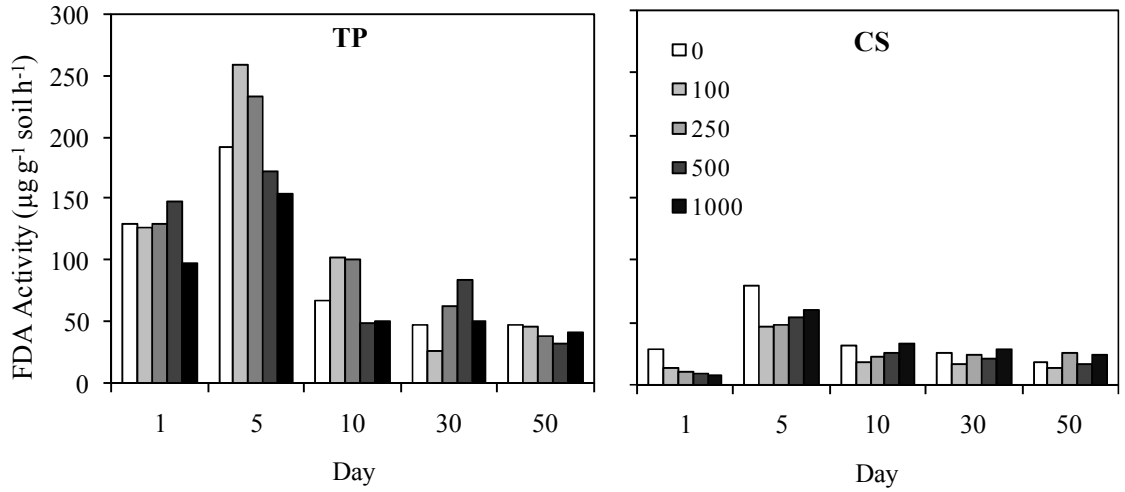

Figure 2. Fluorescein diacetate (FDA) activity of native prairie (TP) and corn-soybean rotation (CS) soils at 1, 5, 10, 30 and 50 days after application of 0 to 1000 PPM concentrations of glyphosate. Only two sites and five selected concentrations are shown.

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Microscale distribution and function of soil microorganisms in the interface between rhizosphere and detritusphere

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Abstract
We used a three-compartment pot design to study microbial community structure and enzyme activity in the interface between rhizosphere and detritusphere. All three compartments were filled with soil from a long term field trial. The two outer compartments were planted with maize (root compartment) or amended with mature wheat shoot residues from a FACE experiment (residue compartment). Soil, residues and maize differed in ^{13}C signature ($\delta^{13}C$ wheat residues -44.1‰, soil -26.5‰ and maize roots -14.1‰) which allowed following the transfer of residue and root-derived C into microbial phospholipid fatty acids. In the interface between rhizosphere and detritusphere, activities of β-glucosidase, xylosidase and phosphatase and the abundance of bacterial and fungal PLFAs were higher in the first 1-2 mm of the root and residue compartment, with generally higher activities in the vicinity of the residue compartment. The $\delta^{13}C$ of the PLFAs suggests that incorporation was limited to the first 1 mm from the residue or root compartments with residue-derived C being incorporated by the soil microorganisms to a greater extent than root-derived C.

Key Words
^{13}C, C flow, enzymes, maize, microbial community structure, roots, wheat

Introduction
The soil influenced by the roots (rhizosphere) and the soil surrounding plant residues (detritusphere) are characterised by high concentrations of easily available compounds and therefore are hot-spots of microbial activity. Close to the roots or residues, microbial density and activity and enzyme activity are high and decrease with increasing distance; forming distinct gradients in mm scale. Moreover, microbial community structure changes with distance from roots or residues (e.g. Kandeler *et al*. 2001, Poll *et al*. 2006). Properties of the rhizosphere and detritusphere have been studied extensively, but separately. However, in the field, roots usually grow in the vicinity of decomposing plant residues. Therefore, it is important to study the interface between rhizosphere and detritusphere on a mm scale. We used a three-compartment pot system with maize on one side and soil with wheat residues on the other, each separated from the 5 mm middle compartment by a 50 µm mesh. Soil, plant and residues differed in ^{13}C signature. The middle compartment was sliced into 1 mm sections. In each section, enzyme activity and microbial community composition by PLFA were measured. Using the differential ^{13}C signatures, we tracked residue or root-derived C in the PLFAs. We hypothesised that: (i) the gradients in enzyme activity and microbial community structure of rhizosphere and detritusphere overlap leading to intermediate values in mid-distance from roots and residues, or (ii) the gradient from either roots or residues dominates, diminishing the gradient from residues or roots, respectively.

Methods
Experimental set up
The experiment was conducted in a three compartment pot system with two outer compartments, each separated by a 50 µm mesh from a 5 mm wide middle compartment. The outer compartments were filled with 664 g and the middle compartment with 99 g dry soil equivalent.
The soil was an agricultural top-soil (Chernozem; C_{org} 1.4%; pH ($CaCl_2$) 7.0; P_{CAL} 93 mg kg^{-1}; N_{total} 0.06%; K_{CAL} 137 mg kg^{-1}; soil texture: sand 12%, silt 66%, clay 22%) from a long-term field trial in Bad Lauchstädt (Germany) with rotations including only C3 plants. Soil was sieved to 2 mm and filled in all three compartments. The mature wheat residues were obtained from a Mini-FACE experiment conducted at the University of Hohenheim (Erbs and Fangmeier 2006). The shoot residues were cut into 2 cm length and mixed thoroughly into the soil of one outer compartments at a rate of 7.5 g kg^{-1} dry soil. Pre-germinated maize (*Zea mays*, cv Amadeo) was planted in the other outer compartment. The $\delta^{13}C$ values were: wheat -44.1‰, soil -26.5‰ and maize roots -14.1‰. There were four treatments which differed in configuration of the outer compartments: unamended soil-un-amended soil (Soil-Soil), maize-un-amended soil (Maize-Soil),

maize-soil with wheat residues (Maize-Residue), soil with wheat residues-un-amended soil (Residue-Soil). The pots were sealed and placed in a water bath in order to maintain the soil temperature at 20°C. The water bath was situated in a glasshouse with ambient light and temperature (summer). Soil moisture was maintained by weight daily.

Sampling
Four replicates of each treatment were harvested on day 14 and 23 after planting (DAP) of the germinated maize seeds. The middle compartment was frozen at -20°C and sliced into 1 mm vertical slices using a kitchen knife. The soil from each 1 mm slice was analysed separately. The soil from the slices and the outer compartments was stored at -20°C until analyses.

Analyses
The activities of β-glucosidase, N-acetyl-ß-glucosaminidase, xylosidase, phosphatase and leucin aminopeptidase were measured using 4-methyl-umbelliferone- or 7-amino-4-methylscoumarin-labeled substrates as described in Marx *et al*. (2005). PLFAs from 4 g of soil (four replicates per treatment, slice and harvest) were extracted using the procedure described by Frostegård *et al*. (1993). ^{13}C in the PLFAs was measured by GC-C-IRMS.

Results
Plant shoot and root dry matter increased 2.5 fold from 14 DAP to 23 DAP and did not differ significantly between the treatments. On 14 DAP, maize had formed a few roots close to the mesh of the middle compartment; by the second harvest, 23 DAP, a dense root mat had formed. Moreover, root density was very high in the lower part of the pots, resulting in relatively dry soil, suggesting that although the water content of the pots was maintained by weight, the water did not penetrate sufficiently into the lower part of the pots to compensate for water uptake by the roots.
Particularly on 14 DAP, activities of β-glucosidase, xylosidase and phosphatase were higher in 1-2 mm distance from roots and residue-amended soil, with generally higher activities in the vicinity of the residue-amended soil than of the roots. Figure 1 shows the β-glucosidase activity as an example, with black bars indicating 14 DAP. The gradients did not overlap in the Maize-Residue treatment resulting in a U-shaped pattern of activities in the rhizosphere-detritusphere interface.

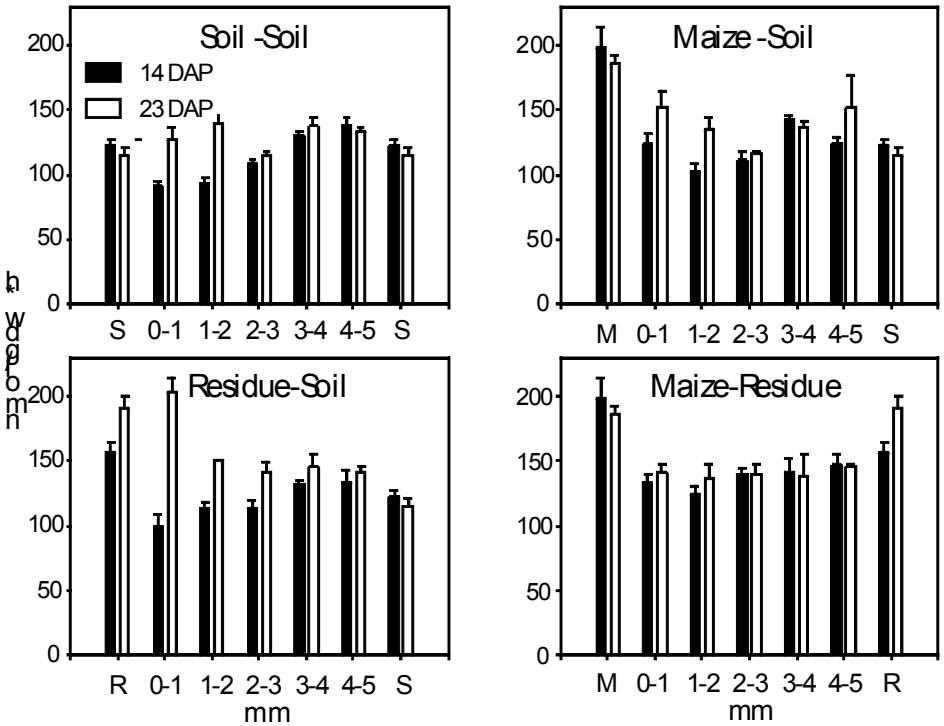

Figure 1. β-glucosidase activity in 1 mm slices of the middle compartment and the outer compartments in Soil-Soil, Maize-Soil, Residue-Soil and Maize-Residue treatments, with S: soil, M:root compartment, R: residue compartment.

The sum of PLFAs, as a measure of active microbial biomass was increased in 1-2 mm distance from the roots and the residue-amended soil with approximately 30% greater values in the detritusphere than the rhizosphere. Compared to the un-amended soil, the concentration of bacterial, Gram-negative and Gram-positive bacterial fatty acids were not increased in the rhizosphere, but were nearly two-fold increased in 1-2 mm distance from the residue-amended soil. The most distinct gradient was found for fungal fatty acids, with 5-7 fold greater concentrations in the vicinity of roots and residue-amended soil than in the un-amended soil (Figure 2). Both roots and residue-amended soil strongly increased the fungal/bacteria ratio up to 4 mm distance. Gradients were generally more distinct at 14 DAP than at 23 DAP. In the Maize-Residue treatment, there was no overlap of gradients for bacterial and fungal fatty acids; however the gradients overlapped for the fungi/bacteria ratio, resulting in increased ratios compared to the un-amended bulk soil throughout the interface.

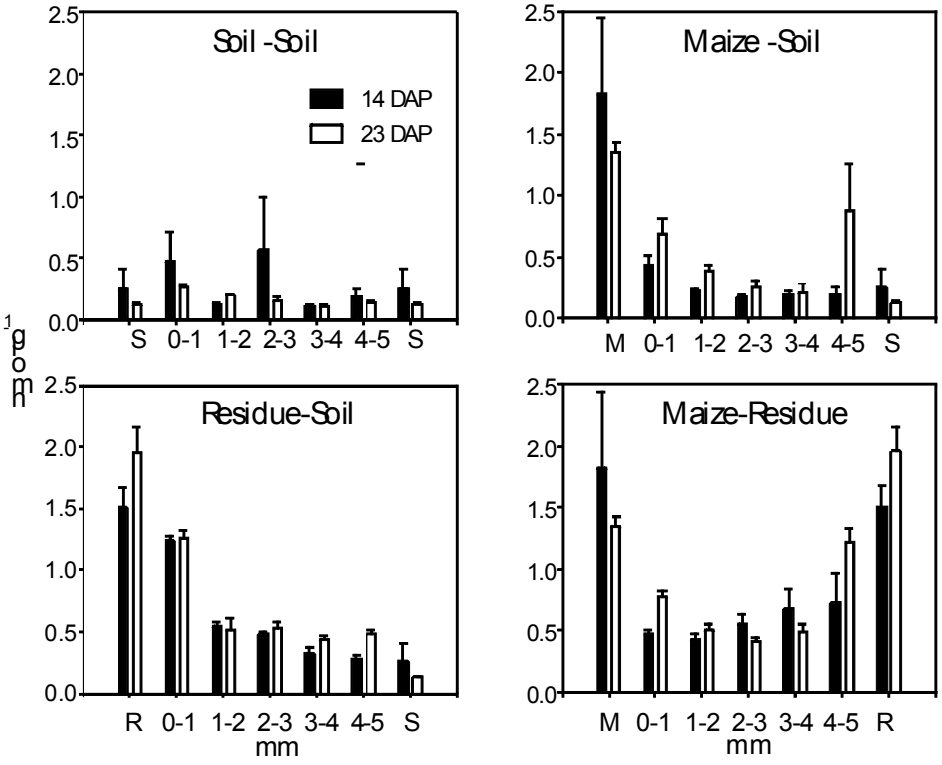

Figure 2. Abundance of fungal PLFA (nmol g^{-1}) in 1 mm slices of the middle compartment and the outer compartments in Soil-Soil, Maize-Soil, Residue-Soil and Maize-Residue treatments, with S: soil, M:root compartment, R: residue compartment.

The $\delta^{13}C$ values of the PLFAs were only determined for the plant-residue amended soil and the residue-unamended soil treatments. Of all PLFAs, only i15:0, 16:1, cy17:0/17:1/i17:0, C18:0 and C18:2ω6/C18:1ω9 showed gradients in $\delta^{13}C$ values (Figure 3 shows C18:2ω6/C18:1ω9). The $\delta^{13}C$ values in 1 mm distance from the residue-amended soil were depleted strongly compared to those in the soil in greater distance whereas the $\delta^{13}C$ values in the vicinity of the root compartment were only slightly enriched, suggesting that in the 14-23 days studied here, microorganisms incorporated residue-derived C to a greater extent than root-derived C.

Conclusion

The results of this study show, for the first time, microscale gradients in enzyme activity and abundance of bacterial and fungal fatty acids in the interface between rhizosphere and detritusphere as well as carbon flow from residues into soil microorganisms. Interestingly, the effect of the residue compartment was greater than that of the root compartment. This was surprising, given the relatively low rate of residue addition and the large residue particle size. In fact, at harvest, many residue particles were still intact suggesting relatively low decomposition rates. We had expected a greater effect of the maize roots because of the release of easily available compounds in root exudates. High rates of exudation can be assumed because of the high growth rate of the maize (dry matter increased more than 2.5 fold between the two harvests). It is possible that the

gradient in the rhizosphere is much steeper than in the detritusphere. Our earlier study (Kandeler *et al*. 2002) suggested that enzyme activities were strongly enhanced in up to 0.5 mm distance from the roots. Since in the present experiment the slices were 1 mm thick, this rhizosphere soil may have been diluted with bulk soil.

The results suggest that the influence zones of root and residue compartment overlapped very little which may be due to the thickness of the middle compartment. To investigate the interface more closely, residue and root compartment may need to be separated by 2-3 mm or less.

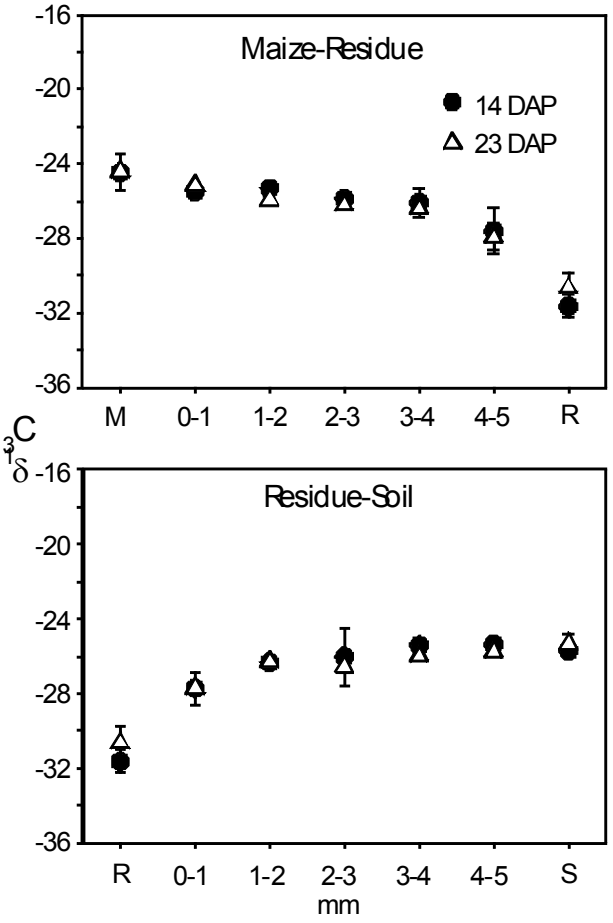

Figure 3. $\delta^{13}C$ in the fungal fatty acids C18:2ω6/C18:1ω9 in 1 mm slices of the middle and the outer compartments in Residue-Soil and Maize-Residue treatments, with S: soil, M:root compartment, R: residue compartment.

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Novel methods to investigate metal interactions with plant cell walls

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Abstract
Low concentrations of some metals markedly reduce root elongation rate and cause ruptures to root rhizodermal and outer cortical cells in the elongation zone. The interactions between metals and plant components responsible for these effects are not well understood but may be linked to changes in water uptake, cell turgor, and cell wall extensibility. Bacterial cellulose (BC)-pectin composites, used as plant cell wall analogs were used as a model system to investigate metal interactions with plant cell walls. Experiments were conducted to examine changes in hydraulic conductivity of BC-pectin composites with metal treatment and the effect of aluminium (Al) on BC-pectin composites uniaxial tensile properties. Hydraulic conductivity of the composites was reduced to ≈ 30 % of the initial flow rate by 39 μM Al and 0.6 Cu μM, ≈ 40 % by 4.6 μM La, 3 μM Sc and 4.4 μM Ru, and ≈ 55 % by 3.4 μM Gd. Aluminium had no measurable effect on the uniaxial tensile properties of the BC-pectin composites.

Key Words
Cell wall, bacterial cellulose, aluminium, toxicity, tensile testing, hydraulic conductivity

Introduction

The toxicity of cationic trace elements to plant roots increases with decreasing soil pH, which is particularly known to lead to aluminium (Al) toxicity in acid soils since Al constitutes ca. 7 % of mineral soils. Toxicities of other trace metals are less common due to low concentrations in parent materials, except in some instances. A distinct symptom of metal toxicity is inhibition of root elongation, and the consequent development of a stunted root system. It has been extensively hypothesised that metals interact with the cell wall through interactions with pectin (Blamey 2003; Kopittke *et al.* 2008; Wehr *et al.* 2004) which makes up 30 % by weight of primary plant cell walls. The apoplast, which is composed of cell walls and inter-cellular spaces, constitutes 5 % or less of root tissue volume but it is important for uptake and transport of water, nutrients, and growth regulating components. The direct consequences of the interactions of metals with the cell wall are not well understood and hotly debated. Physical properties of primary cell walls of plants are difficult to investigate due to their small cell size and heterogenous composition. Yet, BC-pectin composites in their natural hydrated state (typically more than 90 % water) mimic the hydration state of primary plant cell walls, and provide a useful model system for plant cell walls.

Methods

Bacterial composite preparation and characterisation

Gluconacetobacter xylinus strain ATCC 53524 from the American Type Culture Collection (Manassas, VA, USA) was used to form a pectin composite or pellicle (Chanliaud and Gidley 1999). The bacterial strain was cultured in modified Hestrin and Schramm (HS) medium containing 5.5 g l^{-1} peptone, 5.5 g l^{-1} yeast extract, 11.4 g l^{-1} potassium hydrogen phthalate, 0.16 g l^{-1} NaOH and 2 % (w/v) glucose (Hestrin and Schramm 1954). Citrus pectin (Sigma-Aldrich, Australia) (0.5%), de-esterified via alkali treatment to degree of esterification of 33%, and 12.5 mM $CaCl_2$ was added to this medium (initially at pH 4.0). Static incubations were performed at 30 °C for 96 h in 70 mL sterile specimen jars with a diameter of 42 mm, with the resulting pellicle floating on the surface of the medium. The BC-pectin composites were then harvested, gently shaken to dislodge some of the embedded cells, and rinsed in ice-cold 12.5 mM $CaCl_2$ under gentle agitation. Pellicles were stored at 4 °C in 0.02 % NaN_3 until required (i.e. within 2 weeks).

Pectin incorporation into the pellicle (dry weight basis) was determined using the modified colorimetric assay for galacturonic acid (Filisetti-Cozzi and Carpita 1991) after prehydrolysis for 1 h in concentrated H_2SO_4-borate at 0 ºC. The chromophore 3-phenylphenol was used, and sulfamic acid was added as the colour depressant for neutral sugars. The BC-pectin composites all consisted of approximately 30 % pectin by weight, (data not shown) which is consistent with optimum composite formation (Chanliaud and Gidley 1999) and also reflects the pectin content of primary plant cell walls.

Hydraulic conductivity measurements

The concentrations of metals selected were those found to reduce elongation of cowpea (*Vigna unguiculata* L.) roots by approximately 50 %; 30 μM Al, 0.3 μM Cu, 2 μM La, 1.2 μM Gd, 1.3 μM Ru and 1.8 μM Sc (Kopittke *et al.* 2008; Kopittke *et al.* 2009). To measure saturated hydraulic conductivity, pellicles were placed in a flow cell (Figure 1). The glass frit funnel, flow cell and burette was filled with 1 mM $CaCl_2$ solution (ca. pH 5.4), and the burette inserted through the top rubber seal of the flow cell. Thereafter, the burette stopcock was opened and the flow rate through the pellicle was measured by recording the level of $CaCl_2$ solution in the burette every 5 min for 45 min to determine initial flow rate. Then, 5 ml of either 1 mM $CaCl_2$ (controls, pH ≈ 5.4) or metal chloride solution adjusted to pH 4.0 was injected through the rubber septum at the side of the flow cell using a hypodermic syringe. The flow rate was then recorded every 5 min for another 45 min. The treatments were replicated four times. Hydraulic conductivity, *K,* was calculated using the falling head method (Klute and Dirksen 1982) (1);

$$K = \frac{aL}{At}\log_e\left(\frac{h_1}{h_2}\right) \quad (1)$$

with *A*, cross sectional area of the sample; *L*, thickness of the specimen; *t*, time and *a*, the cross sectional area of the burette. These remained constant for each test, allowing the percentage difference in *K* to be calculated from the log of the ratio of initial and final head (h_1 and h_2). Solution within the flow cell was sampled at the end of the experiment (90 min) and analysed via ICP OES or ICP MS (no solution analysis was performed for Al).

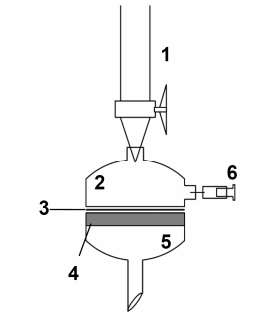

Figure 1. Schematic diagram of flow cell. A standard burette (1) attached to the inlet side of the flow cell (2). The BC-pectin pellicle (3) sits on top of the glass frit (4) which is part of the outlet side of the flow cell (5). A clamp is used to secure the pellicle between (2) and (5). A syringe is inserted through a sealed opening (6) to inject the metal solution at the start of the second 45 min period and to sample solution at the end. The diameter of the flow cell was 10 mm.

Tensile testing

After overnight equilibration in 500 mL of 40 μM, 80 μM or 160 μM $AlCl_3$ in a background of 12.5 mM $CaCl_2$ at pH 4.0, BC-pectin composites were removed from the beakers and tensile tested. Mechanical properties of hydrated bacterial cellulose-pectin pellicles were assessed by uniaxial tensile testing using an Instron 5543 (Instron, Melbourne, Australia) as in McKenna *et al.* (2009). Briefly, dumbbell shaped strips (of known dimensions) were cut using a dumbbell press (ISO 37-4). Three dumbbells were cut per pellicle, five pellicles were tested for each treatment. The two ends were placed directly between vice grips, and moved apart at a constant speed of 10 mm/min. A 5 N load cell was used to record the force required for extension as a function of time. From the geometrical measurements, force-deformation data were converted to stress-strain profiles. Engineering stress (σ) was calculated by F/A where A is the area measured and F is the force in N. Strain (ε) was calculated by ΔL/Lo where ΔL is exerted extension from the starting point Lo, and converted to a percentage. Data was plotted as stress/strain profiles (Figure 2(a)) for each treatment.

Results and discussion

Hydraulic conductivity

All metal treatments caused a significant reduction in hydraulic conductivity compared to the control ($P < 0.001$), with hydraulic conductivity in the control (Ca) treatment reduced to only 80 % (Table 1). However, the injection of metals into the flow cell reduced hydraulic conductivity to approximately 40 % for Sc and La, 45 % for Ru, 55 % for Gd, and to approximately 30 % for both Al and Cu (Table 1). We hypothesise that the observed reduction in hydraulic conductivity following metal treatment was a result of conformational change of the pectic fraction of the composite. Scanning electron micrographs (results not shown) indicated differences in the structure of the composites at the end of the experiment (McKenna *et al.* 2009a).

Metal interaction with the pectin component of the composite is expected to take place either by the exchange of the metal ion tested with Ca^{2+} (as the reticulating cation of the gel), or by the adsorption to any protonated negative charges within the system. The high background of Ca within the system (see Table 2), meant it was not possible to distinguish between exchange or adsorption reactions. It would be expected that carboxyl groups within the pectin would be the predominant source of available binding sites. The magnitude of the reduction was similar for all metals. However, the concentrations of metals causing this reduction were very different. The metals, and their concentrations were selected based on the concentrations causing a 50 % reduction in root elongation in Kopittke *et al.* (2008; 2009). Similar levels of reduction in hydraulic conductivity seen across the trivalent metals suggest a relationship between these metals, which may contribute to the 50 % reduction in root elongation.

Table 1. Metal effects on relative saturated hydraulic conductivity and metal concentration in solution and in the composite at the completion of the experiment.

Metal and concentration (µM)	Relative flow rate (flow rate during second 45 min period relative to the first 45 min period) (%)*	Average metal concentration in flow cell after 90 min (µM)	Average metal concentration in digested pellicle (sampled at end of 90 min) (µg/g)
Al 39	28.3 a	Not measured	Not measured
Cu 0.6	29.3 a	Not detected (< 0.16 µM)	Not detected (< 10 µg/g)
La 4.6	41.9 ab	4.00	55
Sc 3.0	42.5 ab	1.10	8.0
Ru 4.0	45.3 ab	1.39	94
Gd 3.4	54.5 b	1.48	7.7
Ca (control) 1000	80.8 c	1000	52000

*For the mean percentage change in flow rate, means with same subscript are not significantly different at P <0.001 (LSD = 19.5).

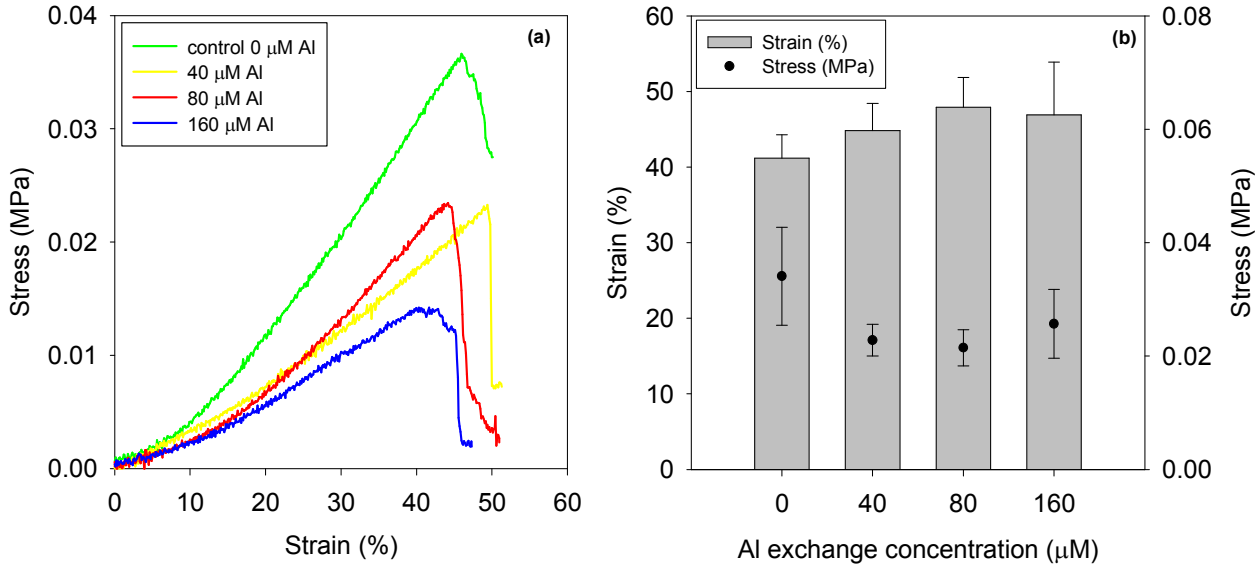

Figure 2. (a) Example stress versus strain plots for control BC-pectate pellicles and three Al treatments as indicated on the graph. (b) Average stress (scatter plots) and strain (bars) values at the three Al concentrations.

Tensile testing

Overall, the presence of Al, at the concentrations tested, had no significant effect on the tensile properties of BC-pectate composites (Figure 2(b)). The current widely-accepted cell wall model (for Type I cell walls) depicts the plant cell wall containing three structurally independent but interpenetrating networks (Carpita and Gibeaut 1993). In this model pectin is not considered a 'load-bearing' component, instead this function is fulfilled by the cellulose-xyloglucan 'scaffolding' network. Cellulose is able to reinforce cell walls under stress by orientating in the direction of stress and it is the mean orientation of cellulose that has been shown to determine wall mechanical properties (Kerstens *et al.* 2001). Chanliaud *et al.* (2002), using BC-pectate composites showed, that upon removal of the pectin component of the composite, tensile deformation profiles remained largely the same, indicating that cellulose was the main contributor to the tensile strength of the composite. However, the presence of pectin modified the arrangement of the cellulose microfibrils as they were being deposited which is analogues to the deposition of the plant cell wall (Chanliaud *et al.* 2002).

The results of the current study further support these past findings, because despite changes in the pectin component of the composite, by the addition of Al, no effect was evident on the stress and strain profiles. However, it is still likely that changes to the pectin component of the composite occurred, through the adsorption of Al. Mimmo *et al*. (2005), showed, with FT-IR, the adsorption of Al onto preformed Ca-pectate gels weakened the overall structure of the gel. Modifications of pectin within plant cell walls has shown to have an impacts on the strength of the cell wall, by modulating access of wall-modifying enzymes to load bearing hemicellulose and cellulose fraction (Ben-Shalom 1986).

Conclusions

Bacterial cellulose-pectin composites have been used, for the first time, as a model system to study metal interactions with plant cell walls. Metal interactions with the pectic component of the composite showed a marked decrease in hydraulic conductivity but the magnitude of reduction was similar for all metals suggesting a relationship between the metals which may contribute to the 50 % reduction in root elongation they cause in plant roots. There was no change in the tensile properties of the composites, after treatment with Al. This finding is consistent with the current cell wall model in which cellulose is the major load bearing cell wall component, and hence changes in the pectic component (through binding of Al) is unlikely to have an impact on uniaxial tensile properties. BC-pectin composites offer a novel system to further investigate hypotheses about metal interactions with plant cell walls.

Acknowledgment

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PCR-DGGE analysis after Bioleaching Stimulation by Indigenous Microorganisms in Vineyards Soil and Copper Mining Waste

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Abstract
Bioleaching techniques can be used to remove copper ions from contaminated soils and recover polluted areas *in situ* or *ex situ*. Several treatments with HCl, H_2SO_4, and $FeSO_4$ were used to stimulation of bioleaching in two experimental conditions applied in vineyard soil and in waste copper mining. The bioleaching treatment using $FeSO_4$ and H_2SO_4 mixed solution had more effect on copper lead, and the second condition using controlled temperature at 30ºC and no water addition to collect soil solution, was more efficient than the first condition using room temperature deionized water to extract soil solution. The treatment with $FeSO_4$ and H_2SO_4 - in the second condition, bioleached more than 1,100 µg/kg of copper from vineyard soil in one collects. After the bioleaching period (115 days), both substrates were analyzed to evaluate the effect of treatments on soil microbial by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA fragments. A high diversity of bacterial was found in the vineyard site as *Rhodobacter* sp., *Silicibacter* sp., *Bacillus* sp., *Paracoccus* sp., *Pediococcus* sp., *Myxococcales* sp., *Clostridium* sp., *Thiomonas* sp., *Firmicutes* sp., *Caulobacter vibrioides*, *Serratia* sp. and Actinomycetales; in the copper mining waste basically was found *Shingomonas* sp. specie in most of treatments; it also was found *Paracoccus* sp. and *Enterobacter* sp. in this soil sample.

Key words
Bioremediation; copper bioleaching; DGGE; microbial community.

Introduction
Soil pollution with heavy metals by either industrial or agriculture activity has been a serious environmental problem. Copper is an essential nutrient for all organisms. However, an excessive concentration of this metal is toxic for most of all living organisms. Vineyard areas are currently potential areas to copper contamination due long-term treatment diseases with copper based fungicide. Copper mining areas increase the copper contamination of adjacent areas as well as produce high amount of waste. The waste is disposed in areas promoting high copper concentrations and consequently environmental pollution. Contaminated areas by heavy metals must be recovered and a remediation processes are necessary to address environmental pollution. Bioremediation is eco-friendly and costly appropriate to this case. In bioremediation process some techniques as bioleaching and biohydrometallurgy has been used to either heavy metal extraction or decontamination (Halinen *et al.* 2009). Bioleaching is an emerging technology with significant potential to add value to the mining industries so as to deliver attractive environmental and social benefits to all associates (Pradhan *et al.* 2008). The DGGE technique is one powerful and ecology tool that has been successfully used to investigate the predominant microorganisms in different environment sites tool (Halinen *et al.* 2009). Furthermore, identification of these microorganisms as well as characterization of environmental population in these areas might be useful in the future to either bioremediation or decontamination of copper polluted areas by efficient microorganisms. For these reason, an environmental conditions to copper bioleaching was created in two different soils being a 40 years vineyard and a copper mining waste area from Southern Brazil. In these soils were used acids, iron, mixture of treatments and environmental conditions to bioleach copper *ex situ*, and after that, it was evaluated the microbial community by DGGE technique.

Methods
The soils were sampled from two copper contaminated soils in Southern Brazil. An Inceptisol was sampled from vineyard area located in Bento Gonçalves, RS, and the other substrate was sampled in the copper mining waste area, located in Caçapava do Sul, RS. The soils were sampled, drayed, sieved (3 mm mesh), homogenized and then weighted 140 g into a 200 mL double plastic flasks used as experimental unit. After

the samples weighted, it was added 10 mL of each treatment (Table 1) into soil and the moisture was adjusted to 80% of field capacity with deionized water.

Table 1. Treatments solutions applied in two periods for copper bioextraction from two different soils.

Treat.	Soil	Treatment Solutions	Extraction Solution	
			1st condition	*2nd condition*
N1	Inceptisol	H_2O (deionized)	H_2O	H_2O
N2	Inceptisol	HCl (0.01%)	H_2O	HCl
N3	Inceptisol	H_2SO_4 (0.128%)	H_2O	H_2SO_4
N4	Inceptisol	$FeSO_4$ (1.35 mM)	H_2O	$FeSO_4$
N5	Inceptisol	$FeSO_4$ (1.35 mM) + H_2SO_4 (0.128%)	H_2O	$FeSO_4$+H_2SO_4
N6	Inceptisol	No treatment- original soil	-	-
R1	Waste	H_2O (deionized)	H_2O	H_2O
R2	Waste	HCl (0.01%)	H_2O	HCl
R3	Waste	H_2SO_4 (0.128%)	H_2O	H_2SO_4
R4	Waste	$FeSO_4$ (1.35 mM)	H_2O	$FeSO_4$
R5	Waste	$FeSO_4$ (1.35 mM) + H_2SO_4 (0.128%)	H_2O	$FeSO_4$+H_2SO_4
R6	Waste	No treatment-original waste	-	-

The experiment was conducted in two different conditions. In the first condition, the soil moisture of all treatments was adjusted with deionized water and incubated at room temperature. The soil solution was obtained by the displacement column using deionized water. In the second condition, the soil moisture was adjusted with the treatment solutions and incubated at controlled temperature (30ºC ± 1) (Table 1). It was added 30 mL of either water or treatment solutions into the samples; this was realized according to each experiment condition. This process was taken 8 hours. After soil solution collect, the solution pH and copper content in soil solution were measured. After the measurements of all soil solutions, the soil samples were collected and stored in freezer (-4ºC) until the DNA extraction. The DNA extraction of soil was done for all treatments with the UltraCleanTM Soil DNA Isolation Kit (MOBIO, USA), using 0.5 g of soil sample of each treatment. After DNA extraction, it was amplified for PCR reactions with a CG clamp primer 338F (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GACTCCTACGGGAGGCA-3') and 519R (5'-GWATTACCGCGGCKGCTG-3'). The DGGE analysis was performed in a DCDE Universal Mutation Detection System (Biorad, Hercules, CA, USA) apparatus. The PCR product generated by 338F-GC and 518R primers were loaded onto 6% (w/v) polyacrylamide gels in 1x TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) buffer, 1 mm thick and 16 x 16 cm sized. The polycrylamide gel was made with linear denaturating gradient of urea and formamide ranged from 40% to 60% (where 100% denaturat contains 7 M urea and 40% formamide) by gradient Marker (Bio-Rad, Hercules, CA, USA). The electrophoresis conditions were run for 4 h at 200 W in 1x TAE buffer at a constant temperature of 60ºC. The gel plate was cooled in ice water for 10 min., and the gels were stained in an ethidium bromide solution (0.5 µg/mL) for 15 min., and distained in 1 x TAE buffer for more 15 min. The distained gel was placed in a UV trans-illuminator and digitalized using a digital camera. The target band was removed from the DGGE gel and was placed into a 1.5 mL sterile tube containing 20 µL of sterilized water. The freeze-thawing cycle was performed thrice at -80 and 50ºC and 1 µL of eluted DNA was amplified with primers 338F and 519R. The PCR conditions have been described in the preceding section. PCR was performed as previously described and the analysis of DNA sequences and homology searches were completed using the BLAST algorithm for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn). Genbank BLAST (N) was used for homology searches.

Results

Bioleaching with different treatments in vineyard soil contaminated with copper (Figure 1A) and copper mining waste area was analyzed (Figure 1B) was evaluated. All treatments had the same tendency in the first experimental condition, when they were compared with water treatment (N1). The treatments on the fifth collect that received acid N2, N3 and N5 were better than the other treatments on copper bioleaching with 21.44, 82.56 and 59.91 mg of copper per kg of soil respectively. In addition, the copper bioleaching concentration in all acid treatments in the second condition were superior than 11 mg/kg of soil, and the

highest copper extraction occurred with the fifth collect in the treatment with H_2SO_4 (N3) with more than 82.56 mg/kg of copper bioleached from soil after 115 days of incubation. In the other hand, the first condition did not go over 1.42 mg of copper per kg of soil bioleached (N5) (Figure 1A). The treatments with sulfuric acid H_2SO_4 (R3) presented copper bioleaching of 2.60 mg/kg in the 4th collection, and the treatment with iron and acid together ($FeSO_4$ and H_2SO_4 (R5)) with 15.08 mg/kg and 20.12 mg/kg showed high copper bioleaching in the 4th and 5th collection respectively, when compared with the other treatments in study. Also, the second condition of incubation showed better results in copper bioleaching in copper mining waste (Figure 1B).

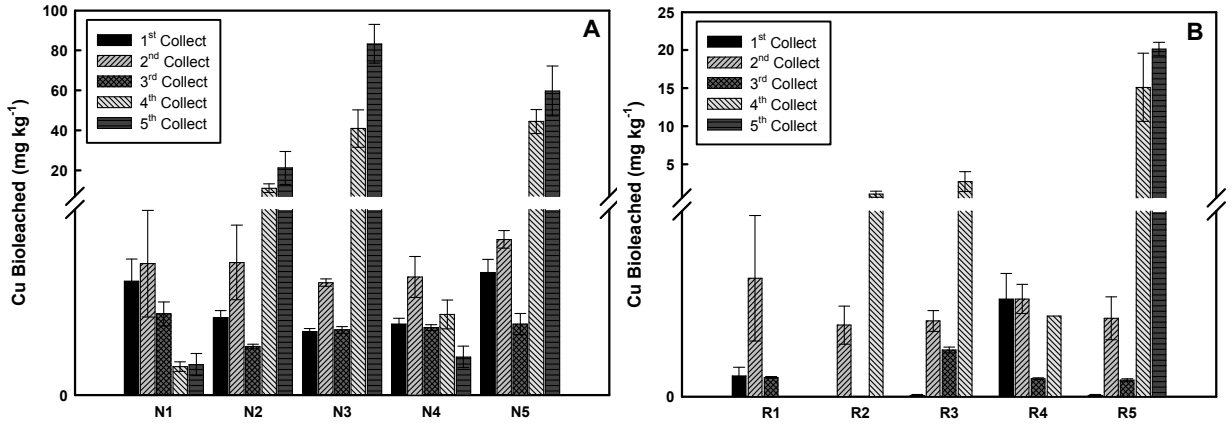

Figure 1. Extraction of copper from Inceptisol (mg of Cu per kg of soil) with different treatments: N1=H_20, N2=HCl, N3=H_2SO_4, N4=$FeSO_4$ and N5=$FeSO_4$+H_2SO_4 (A); and copper mining waste with different treatments: R1=H_20, R2=HCl, R3=H_2SO_4, R4=$FeSO_4$ and R5=$FeSO_4$+H_2SO_4. Error bars are standard error of the mean.

Figure 2 shows the DGGE profiles indicating the number of bands detected at each sample and matched bands among lanes. It shows that number of bands detected at each well are 4 in the vineyard soil (1. Inceptisol) and 2 in the in the copper mining waste (2. waste), where just the treatment with HCl (R2) had just the band A in the well and the others treatments had bands A and B.

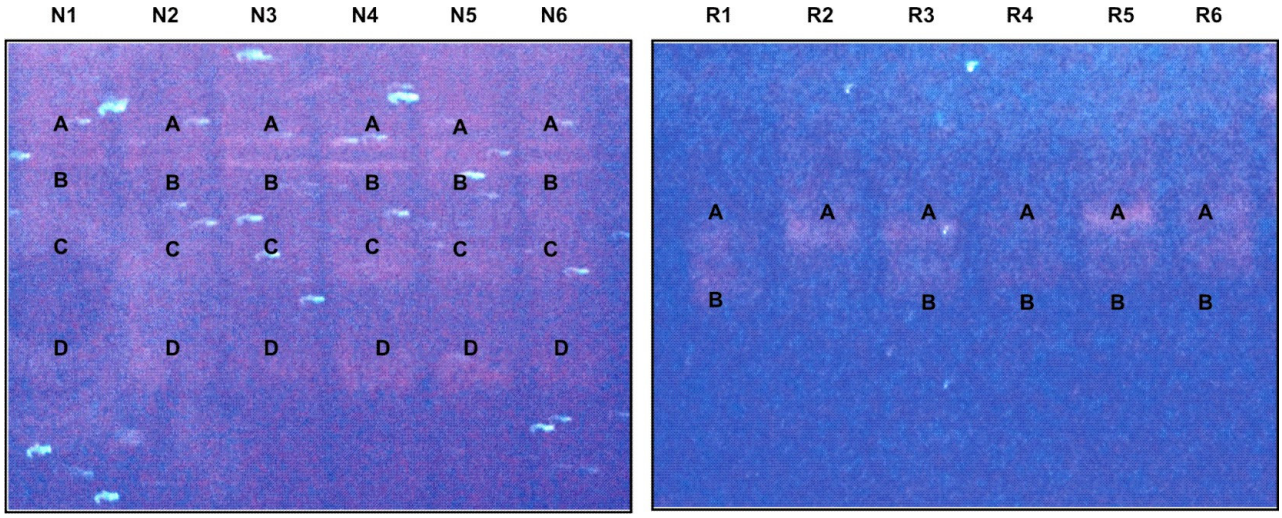

Figure 2. Representation of the DGGE profiles, where picture 1 is from Inceptisol and picture 2 is from copper mining waste area.

Thirty-five bands were cut and sequenced from DGGE gels of vineyard soil contaminated with copper and copper mining waste (Figure 2), and twenty-eight of these bands were identified by 16S rRNA sequence analysis. Two isolates from vineyard soil were identified as *Rhodobacter* sp. (in the treatment-band N1-A and N4-A), one as *Silicibacter* sp. (N1-B), one as *Bacillus* sp. (N1-C), four as *Paracoccus* sp. (N1-D; N2-D; N3-D and N4-D) all in the band D, one as *Pediococcus* sp. (N2-A), one as Myxococcales (N2-B), two as *Clostridium* sp. (N2-C and N6-D), one as *Thiomonas* sp. (N3-A), one as Firmicutes (N3-B), one as *Caulobacter vibrioides* (N3-C), one as Sphingobacteria (N4-B), one as *Serratia* sp. (N5-A), and one as

Actinomycetales (N6-C). From the copper mining waste, it was identified almost six isolates as *Sphingomonas* sp. (R1-A; R2-A; R3-A; R3-B; R4-A and R5-A), one as Sphingobacteria (R1-B), two as *Paracoccus* sp. (R4-B and R5-B), and one as *Enterobacter* sp. (R6-B).

Discussion

Copper is an essential micronutrient for living microorganisms but at high concentrations is a toxic heavy metal in the environment. Copper microbial bioleaching from contaminated areas (Halinen *et al*. 2009) have been increasing attention in recent years. In this work were used principles of copper bioleaching and biohydrometallurgy to remove copper from two different sites contaminated with copper (40 years of vineyard production and copper mining waste using the stimulation of indigenous community of soil). Furthermore, it was used fingerprinting method (DGGE) to analyze the effect of the treatments on soil microbial community, where it is important to use microbial control methodologies to study in reliable and automatic way the microorganisms associated with different zones of bioleaching process to subsequent optimize their efficiency (Malki *et al.* 2006).

After the treatments, it was extract DNA from samples and used PCR-DGGE method to evaluate the soil microbiota community. It was identified more than one microorganism per band in the PCR-DGGE gel from vineyard soil, i.e. *Silicibacter* sp., Myxococcales, Firmicutes, and Sphingobacteria where found in the band "B" of vineyard soil. Otherwise, it is known that one band could possibly represent more than one species (Heuer and Smalla 1997) and also, some bacteria can produce more than one band on DGGE gel method (Muyzer *et al.* 1993). Comparing our study with other DGGE study with vineyard soil contaminated with high copper concentrations to evaluate the microbial community (Dell'Amico *et al.* 2008), it was verified similar diversity in some species such as *Bacillus* sp. and *Sphingomonas* sp., where they were found in both studies. Also, it was verified that the specie *Sphingomonas* sp. showed in the copper mining waste in abundance in all treatments, less in the control R6 (natural mining waste).

In summary, the second condition applied with controlled temperature and addition of the same treatment to collect soil solution was more efficient to copper bioleaching than the other tratments. In the community of bacteria found after treatments analyzed with DGGE assay, it was found basically gram positive bacteria. Firmicutes filum as *Clostridium* sp. and *Bacillus* sp., was found in the vineyard soil. Other important information was the isolate *Paracoccus* sp., where its pathway there is the oxidation of SO_4^{2-}, where it is important to copper bioleaching. Microorganisms identified as *Sphingomonas* sp. was widely found in the copper mining waste in study, known of the characteristics as metal resistant, they can be used for bioremediation studies especially in this are.

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Phosphorus acquisition characteristics of cotton (*Gossypium hirsutum* L.) plant: a review

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Abstract
This paper provides a review of P acquisition strategies by cotton plants. Except for excretion of phosphatase enzymes, the cotton plant lacks the ability to manipulate its rhizosphere chemistry, and to mobilize non-labile inorganic P sources. Phosphorus acquisition by cotton plants mainly depends on root exploration of relatively labile inorganic P and organic P sources from the surface and subsurface soil layers. Root morphological traits, such as increased root to shoot ratio or AM associations, would result in a high root absorbing surface area. Subsoil P sources could be accessed by cotton plant over time possibly due to the water stress encountered at the topsoil, and the presence of roots in the subsoil. The role that mycorrhizae play with cotton plants in Vertosol soils is worthy of future investigation.

Key Words
Hydraulic lift, P response, Root exudates, Soil depth, Residual P, P placement.

Introduction

Cotton (*Gossypium hirsutum* L.) production is an important agricultural industry in Australia. Lint yields from flood-irrigated cotton crops have increased steadily over the past 25 years and impose a high demand for nutrients (Rochester 2007). Although cotton lint is composed of primarily cellulose, considerable amounts of nutrients can be removed with cotton seed (Dorahy *et al.* 2004; Rochester and Peoples 1998). In Australia, cotton production requires regular P fertilizer inputs (~ 20 kg P/ha) in order to maintain soil P fertility and high lint yields, especially in the last 25 years (Dorahy *et al.* 2004). Nevertheless, the response of cotton to P fertilizers is unpredictable and frequently low. Dorahy *et al*. (2004) reported that only 3 out of 17 cotton field sites in Australia showed increases in lint yield from P fertilizer application. In addition, the reported critical Colwell P concentration for cotton in Vertosols varies from 6 to 12 mg/kg (Dorahy *et al.* 2004; Hibberd *et al.* 1990), which is much lower than those for wheat (21 mg/kg) and barley (18 mg/kg) on similar soils with low P sorption capacity (Reuter *et al.* 1995). This suggests that cotton may be able to access P from stable soil P pools. Such knowledge on P acquisition characteristics of cotton would increase our understanding of P responses by cotton to P fertilizers. This paper reviews possible root strategies adopted by cotton plants in their P acquisition from the soil.

Root morphological and physiological traits

Carboxylates and proton release

Significant exudation of carboxylates was not detected in the rhizosphere of cotton in response to P deficiency (Wang *et al*. 2008). Proton efflux of cotton plant is frequently related to the N uptake as NH_4^+ rather than insufficient P supply. For example, Hylander *et al.* (1999) reported a greater rhizosphere acidification of cotton than maize and soybean when N source was applied as NH_4NO_3. In the case of NO_3^- as the sole N sources, alkalization of the rhizosphere by cotton was detected irrespective of shoot P status (Wang unpublished data). In addition, cotton was not superior in using sparingly soluble P sources such as Al, Fe and Ca phosphates, when compared with both wheat and white lupin (Wang unpublished data). It appears that cotton plants lack the ability to manipulate its rhizosphere chemistry and to mobilize non-labile inorganic P sources, in terms of both carboxylate and proton release. Nevertheless, the enhanced acid and alkaline phosphatase activity in the rhizosphere of cotton could promote the utilization of P from soil organic pools, as demonstrated by the concurrent depletion of NaOH-extractable organic P ($NaOH\text{-}P_o$) in the rhizosphere soil (Wang *et al*. 2008; Figure 1). While Dorahy *et al*. (2004) found a strong correlation between relative P uptake of cotton and Al- and Fe fraction of soil P in a field experiment, these pools in Vertosols normally represent P weakly absorbed with Al and Fe oxides (Holford and Mattingly 1975; Soils and Torrent 1989).

Root to shoot ratio

Like many species, P stress causes a preferential distribution of dry matter and P content to the roots of cotton (Gill *et al.* 2005; Maqsood *et al*. 2005; Wang *et al*. 2008). Indeed, P-deficient cotton plants can retain 35% of total P in its roots, compared with only 14% in P-sufficient cotton (Ahmad *et al*. 2001). Cotton plants have the peak consumption of P later in the growing season (first peak bloom) when the root system is fully developed (Schwab *et al*. 2000), which possibly indicates that P acquisition of cotton mainly depends on its root morphological exploration of labile P sources. Ahmad *et al.* (2001) also found that the tolerance of cotton genotypes to P deficiency was due to their efficiency in absorption of soluble P and P utilization for biomass synthesis. Thus, the greater allocation of assimilates to root growth due to P stress by cotton could confer a significant advantage in soluble P acquisition by cotton.

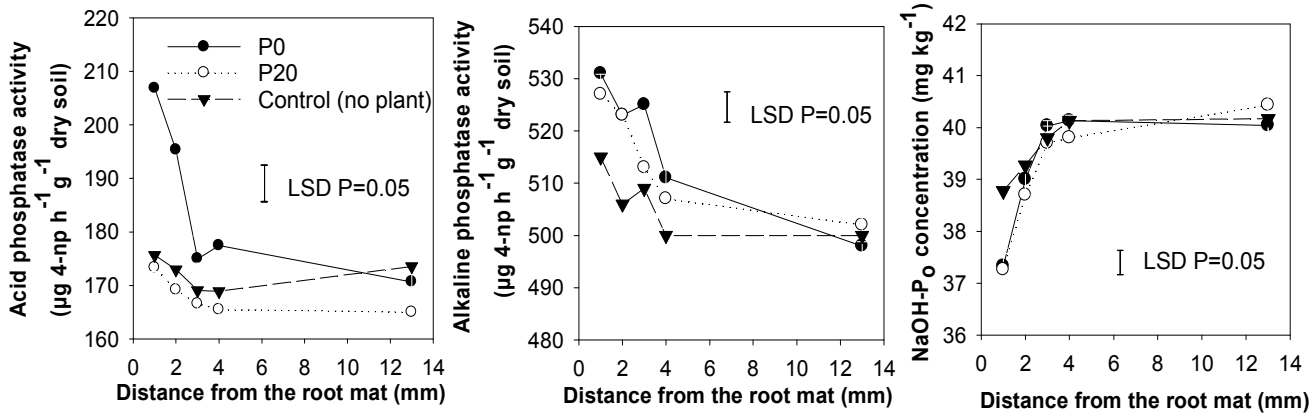

Figure 1. Changes in acid and alkaline phosphatase activity, and concentration of NaOH-P_o with distance from the root mat of cotton with 0 and 20 mg/kg P application. For each panel, the vertical bar indicate the LSD (P=0.05) for the treatment × distance interaction (Wang *et al*. 2008).

AM fungi

Cotton crops grown in Australian are known to be well infected with arbuscular mycorrhizae (AM) fungi (Rich and Bird 1974). The external hyphae of AM fungi contribute to an increased P uptake due to an increased surface area for absorption and decreased distance for P diffusion (Bolan 1991; Schnepf *et al.* 2008). Poor P uptake of cotton on virgin soil in tropical Australia had been related to the lack of an association with AM (Duggan *et al.* 2008). Graham and Syvertson (1985) suggested that plant species like cotton with less branched and coarse root systems could be highly dependent on mycorrhizal association for P acquisition compared to that of species with finely branched roots.

Hydraulic lift

As a tropical species, cotton is normally cultivated in warm to hot climates. Rapid drying of furrow-irrigated soils due to evaporation is quite common under cotton production (Muchow and Keating 1998; Singh *et al.* 2006). Redistribution of water from wet subsoil layers into drier topsoil through plant root systems, a phenomenon known as hydraulic lift, could be a desirable strategy for P acquisition by cotton plants from surface soil that experienced frequent dryness. Hydraulic lift would enhance shallow root survival and P availability at the topsoil (Bauerle *et al.* 2008; Huang 1999). By using gamma densitometry, Baker and van Bavel (1988) detected an overnight movement of water from wet to dry soil through the cotton root system. Wang *et al*. (2009) also demonstrated the occurrence of hydraulic lift by cotton plant grown on a Vertosol. Nevertheless, the detected hydraulic lift did not aid P uptake from the drying topsoil (Wang *et al*. 2009). Higher root mortality and lower P diffusion rate in the Vertosol, compared with sandy soil, could account for the negligible P uptake from water-stressed surface soil (Wang *et al*. 2009).

Phosphorus acquisition depth

Top soil

The cotton seedlings had been reported to derive most of its P from the fertilizer band applied to the top 10 cm layer (Dorahy *et al.* 2008). Nevertheless, at a later growth stage (36 days after sowing), soil P pools beyond the fertilizer band showed a significant contribution (more than 90%) to the total P uptake by cotton (Dorahy *et al.* 2008). Poor responsiveness of cotton to shallow P placement had been attributed to the decreased soil moisture at the topsoil (Hibberd *et al.* 1990; Singh *et al.* 2005), which is in consistent with findings from Wang *et al*. (2009) that P uptake from the Vertosol was strictly regulated by its soil water content.

Subsoil

Approximately half of the root system of the cotton plant lies below the surface 15 cm soil layer (Schwab *et al*. 2000). Under frequent drought conditions, seed cotton yield in northern Australia showed a significant increase (17%-67%) to the P applied in subsurface (10-15 and 25-30 cm deep) over that applied at shallow depth (7-10 cm) (Singh *et al*. 2005). In addition, subsoil P pools e.g. residual and total organic P, showed a depletion following long-term cotton cropping (Wang unpublished data, Figure 2). Exploration of subsoil P by cotton roots may act as an important root morphological adaptation to the water stress-induced unpredictable availability of P at the topsoil. The contribution of subsoil P sources to plant P uptake would depend on many factors, including the moisture level of the topsoil, soil texture and the presence of root and P sources in the subsoil (Kuhlmann and Baumgartel 1991; Wang *et al*. 2007; Wang *et al*. 2009). Deep P placement is effective in increasing cotton yield under field conditions possibly because of an enhanced contact between root and fertilizer during the later stages of growth, and also a sustained P availability under periodic surface drought conditions.

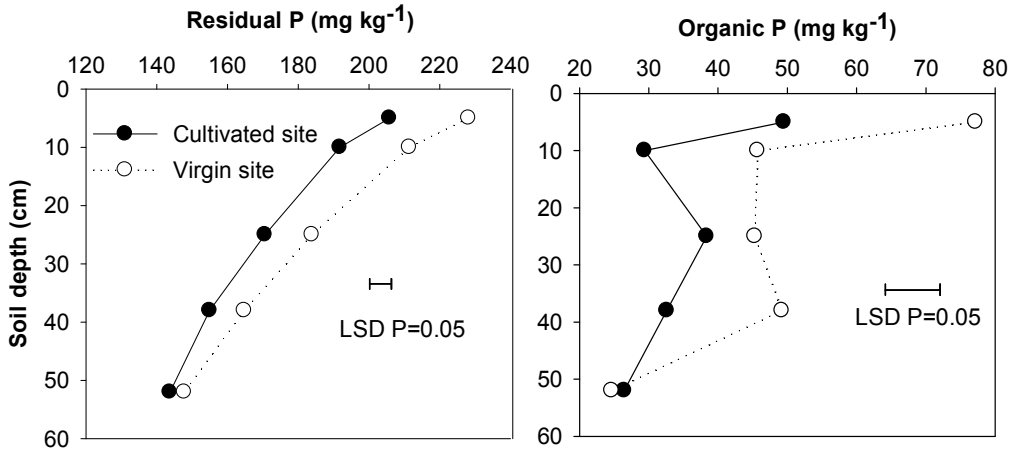

Figure 2. Residual and total organic P concentration (mg/kg) pooled from three sites cultivated with continuous cotton for more than 20 years, and from adjacent three virgin sites, at soil depth of 0-5, 5-10, 10-20, 20-30, 30-45 and 45-60 cm on a Vertosol in northern NSW and southern Queensland. Bars are LSD values at P=0.05 (Wang unpublished data).

Conclusion

Evidences suggest that P acquisition by cotton plants mainly depends on its ability to access relatively labile inorganic P, and organic P from both topsoil and subsoil layers. In this respect, the role that mycorrhizae play with cotton plants in Vertosol soils is worthy of future investigation. Routine soil P tests using alkaline bicarbonate extraction solution (Colwell P) on soil samples collected from the topsoil layers (above 10 cm) do not adequately estimate P responsiveness of cotton to the application of P fertilizers. Low responsiveness of cotton to P fertilizers applied in the soil with low soil test values would indicate that cotton was able to meet their P requirement from the P pools not defined by bicarbonate extractants, such as organic P and subsoil P sources, without the need for P fertilizers.

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Phosphorus ameliorates aluminium toxicity of Al-sensitive wheat seedlings

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Abstract
The role of phosphorus (P) in the amelioration of aluminium (Al) toxicity to plants is still unclear. The aim of this study was to examine the amelioration of Al toxicity by P supply. The study involved growing Al-sensitive wheat seedlings for 13 days in an acidic soil (pH 4.5 in $CaCl_2$) with increasing added rates of P (0, 20, 40 and 80 mg P/kg soil) and Al (0, 50 and 150 mg $AlCl_3$/kg soil). The results indicated that the effects of Al toxicity in this soil could be fully alleviated by the application of P at 50 mg $AlCl_3$/kg. The highest 150 mg/kg $AlCl_3$ treatment significantly reduced root growth, but this was partially overcome by the 80 mg/kg P treatment. High P significantly reduced the concentration of Al in the apoplast, and in the root and shoot. It is possible that an insoluble Al-P complex forms in the soil and this decreases Al bound in apoplast as well as uptake into the roots. High P decreased the translocation of Al from root to shoot.

Key Words
Al tolerance, Al translocation, Apoplast Al, P-Al interactions

Introduction
Aluminium toxicity and P deficiency are two common constraints limiting crop production in acid soils (Liao *et al.* 2006). Aluminium toxicity is considered to be closely associated with the phosphorus nutrition of plants and P may be an effective agent for detoxifying excess Al (Bollard 1983). However, relatively few studies have been done to investigate how improved P nutrition alleviates Al toxicity in plants (Tan and Keltjens 1990). Understanding the mechanisms underlying Al and P interactions will help to develop management options to sustain crop production in acid soils. The objectives of this study were i) to investigate the growth response of Al-sensitive wheat seedlings to increasing P and Al supply in an acid soil, and ii) to understand how P alleviates Al toxicity. We hypothesized that P will ameliorate Al toxicity in both the soil and within the plant.

Materials and methods
Soil, plants, experimental design and procedure
The subsurface layers of an acidic Podosol (Isbell 2002) were used in the experiment. The soil had extractable Al of 4.98 mg/kg, a field capacity of 13% (w/w), a pH buffer capacity of 0.24 cmol/kg/pH, a sand texture (95.2 % sand, 0.4 % silt and 4.4 % clay) and a total organic C of 0.69%. The experiment consisted of 3 levels of added Al (0, 50 and 150 mg $AlCl_3$/kg soil) × 4 levels of P (0, 20, 40 and 80 mg P/kg soil) with 5 replications. Basal nutrients were not applied to minimize the interactions between nutrients and Al in the soil, so plant growth over the 13 day study relied on the seed reserve. $AlCl_3$ was added as a stock solution to soil and the soil was pre-incubated at 30^0C for 7 days. Before sowing, KH_2PO_4 was applied directly to the soil after pre-incubation and thoroughly mixed. The Al-sensitive wheat genotype (ES8) was used as a testing plant. Eight pre-germinated uniform seeds were sown in each replication in plastic cups containing 200 g soil, and subsequently thinned to 6 plants/cup. Plants were grown in a growth cabinet with day/night temperatures 20/18 ^{0}C, 10 h dark and 14 h light conditions, and an average light intensity of 9660 lm-m^{-2}.

Measurements
The experiment was harvested 13 days after sowing. After roots were washed free of soil particles, plant height, and root and shoot biomass were recorded. Root morphological parameters were measured using the WinRHIZO image analysis system (WIN MAC, Regent Instruments Inc., Quebec, Canada). The soil-available Al in the same extract was measured by using a modified pyrocatechol violet (PCV) method (Kerven *et al.* 1989). Apoplastic Al was extracted in 50 mM $BaCl_2$ solution, chilled to 0^0C for 45 minutes and determined spectrophotometrically at 585 nm using the PCV method with standards prepared in the extacting solution (Wang *et al.* 2004). The dried root and shoot samples were digested in HNO_3/$HClO_4$ mixture (4:1,v/v) (Zheng *et al.* 2005). After digestion, root and shoot Al concentrations were determined using the colorimetric method described by Nursyamsi *et al.* (2002). Concentrations of P in the roots and shoots were determined colorimetrically using malachite green (Motomizu *et al.* 1983)

Statistical analysis
Results were analysed by a two-way analysis of variance (ANOVA) using Genstat 5th edn for Windows (Lawes Agricultural Trust, UK).

Results and Discussion

Increasing P supply substantially decreased extractable Al in bulk soil (Figure 1). This decrease in Al extractability in the soil is likely to have resulted from the chemical precipitation of Al with the added P thereby lowering the activity of Al^{3+} in the soil solution (Nakagawa *et al.* 2003; Sanzonowicz *et al.* 1998; Silva *et al.* 2001).

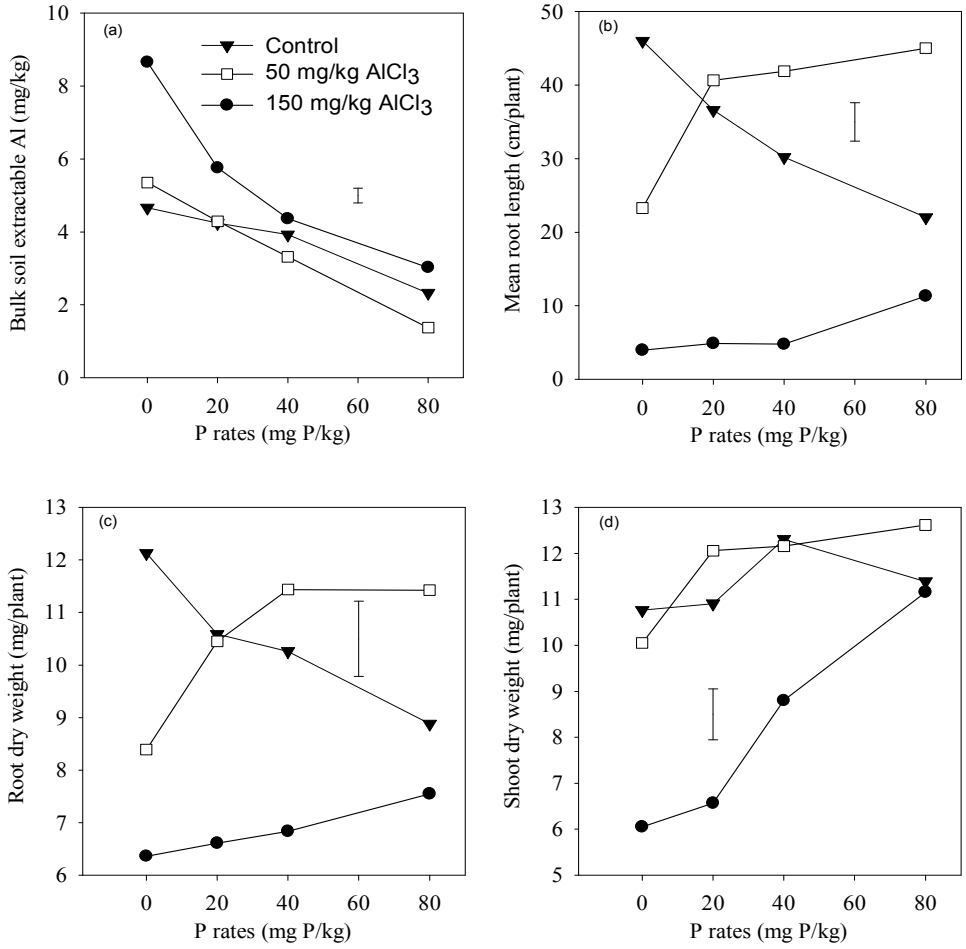

Figure 1. Effect of P supply on (a) extractable Al in bulk soil, (b) root length, (c) root dry weight and (d) shoot dry weight of Al-sensitive wheat seedlings after 13 days of growth. Bar represents LSD (*P*=0.05) for Al×P interaction.

A marked Al by P interaction occurred with the growth of the wheat roots. Increasing P supply resulted in a linear decline in root length and root dry weight in the absence of added Al (Figure 1b, c). With the50 mg/kg $AlCl_3$ treatment, both root length and root dry weight increased significantly (p<0.05) with the 20 mg/kg P treatment and then remained consistently high with further added P. However, the 150 mg/kg Al treatment markedly depressed root growth with nil P; root length and dry weight were then increased by 65% and 16%, respectively with the 80 mg/kg P supply. These results confirm that increasing P supply can ameliorate the toxic effects of Al on root growth root growth, but the extent of the amelioration is dependent on the severity of the toxicity.

High P supply was more effective at ameliorating the effect of Al toxicity on shoot growth than on the roots. Despite the 40% reduction in shoot dry weight with the 150 mg/kg $AlCl_3$ treatment with nil P, there was no difference in shoot dry weight when 80 mg/kg P had been added (Figure 1d). Shoot mass did not differ significantly between the control and 50 mg/kg $AlCl_3$ treatment. Thus there was a very positive effect of

increasing P supply on shoot growth under severe Al toxicity. It has been suggested that this is related to indirect effects associated with nutrient uptake in the plant growth response (Tan and Keltjens 1990).

High P reduced Al in the apoplast and inside roots. With the 80 mg P/kg treatment, apoplast Al was reduced by 72%, 57% and 65% in 0, 50 and 150 mg/kg $AlCl_3$ treatments, respectively. However, total root Al was reduced by only 4%, 22% and 29% with these Al treatments (Figure 2a, b). These results indicate that the exclusion of Al by P was greater in the apoplast than in the roots. In addition, our result showed that apoplast Al was around 37% of the total root Al. Other research showed that apoplastic Al was consistently 30 to 40% of total root Al (Tice *et al.* 1992).

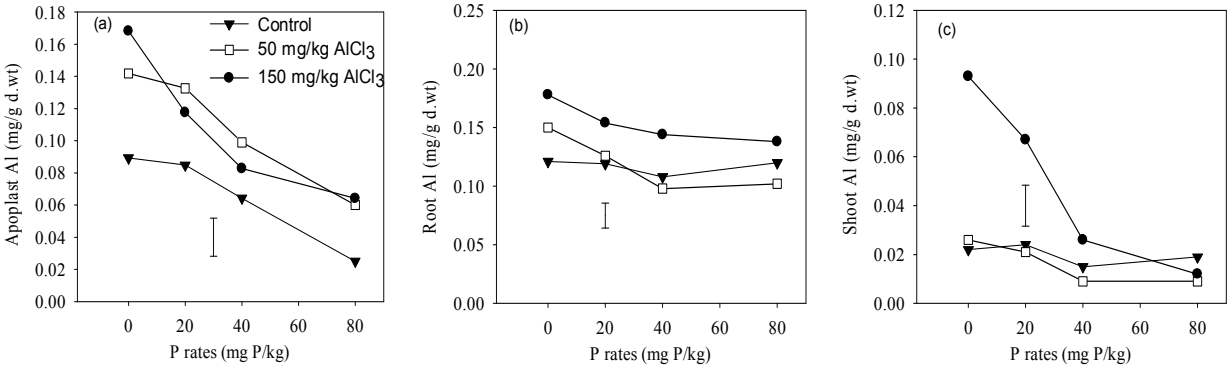

Figure 2. Effect of P supply on Al concentrations in (a) apoplast, (b) root and (c) shoot of Al-sensitive wheat seedlings after 13 days of growth. Bar represents LSD (*P*=0.05) for Al×P interaction.

A key finding was that Al translocation from the root to the shoot with the 150 mg $AlCl_3$/kg treatment, which was 5 times that of the 0 and 50 mg $AlCl_3$/kg treatments with no added P, was markedly reduced with 80 mg P/kg (Figure 2c). This was associated with a significant reduction ($P<0.05$) in shoot P concentration, suggesting a possible causal relationship. Irrespective of the level of added $AlCl_3$, the wheat seedlings were able to take up more P from the soil, translocated more P to the shoots and utilize P more efficiently for shoot growth and development, with increasing P supply, although total P uptake was reduced with the high Al treatment.

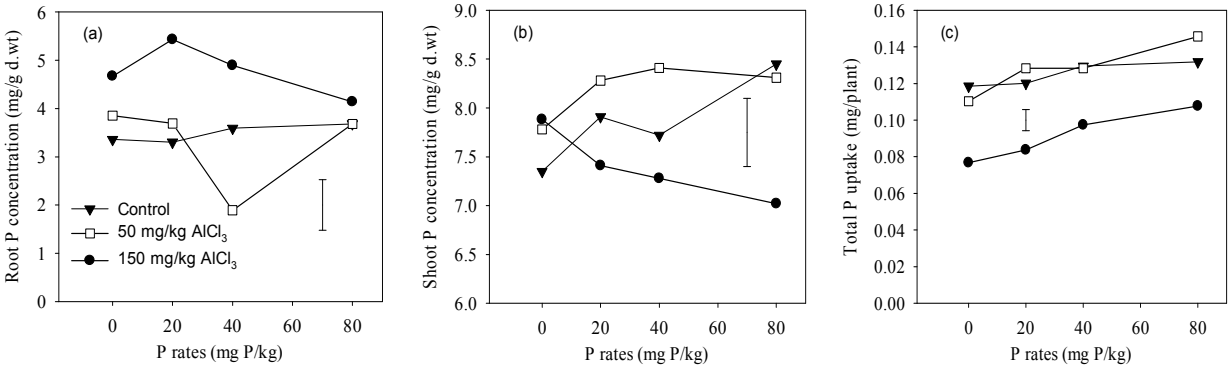

Figure 3. Effect of P supply on P concentrations in (a) root and (b) shoot, and (c) total P uptake of Al-sensitive wheat seedlings after 13 days of growth. Bar represents LSD (*P*=0.05) for Al×P interaction.

The reductions in apoplast root and shoot Al concentrations with increasing P supply shows that high concentrations of soil P can ameliorate the toxic effects of Al on these seedlings (Figure 2). These findings have only previously been found in solution culture studies (Gaume *et al.* 2001; Nakagawa *et al.* 2003).

Conclusions

This study demonstrated that increasing P supply improves the tolerance of these Al-sensitive wheat seedlings to Al toxicity. There are at least four ways in which P alleviates Al toxicity. First, P directly reacted with Al in soil presumably to form Al-P precipitates, and thus lower Al^{3+} activity in soil solution. Second, P decreased the amount of apoplastic Al that was bound to the root cell walls and this binding was around 37% of the total Al uptake by the root. Third, high P supply decreased total uptake of Al into the plants (50%) with the reduction in Al concentration in the roots (12%) being less than that in the shoots (88%) with the high Al treatment. Finally, P decreased the translocation of Al from roots to shoot by up to 90% in high P and high Al supply.

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Plant growth promontory attributes by 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing *Methylobacterium oryzae* strains isolated from rice

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Abstract
Species from the pink-pigmented facultative methylotrophic bacteria (PPFMs) genus *Methylobacterium* are versatile in nature and common inhabitants of plants, potentially dominating the phyllosphere population, but are also found in other organs. The consistent success of the *Methylobacterium*-plant association relies on methylotrophy, the ability to utilize the one-carbon compound methanol emitted by plants. Accordingly, the present study investigated the inoculation effects of *Methylobacterium oryzae* strains CBMB20 and CBMB110 on plant growth and accumulation of phytohormone levels of tomato and red pepper under gnotobiotic conditions. Seeds treated with the *Methylobacterium* strains showed a significant increase in root length when compared with the uninoculated control. Extracts of the plant samples were used for indole-3-acetic acid (IAA), trans-zeatin riboside (*t*-ZR), and dihydrozeatin riboside (DHZR) assays by immunoanalysis. The treatment with *M. oryzae* CBMB20 or CBMB110 produced significant increase in accumulation of cytokinins t-ZR and DHZR in red pepper and tomato plant extracts. Greenhouse experiments further confirmed the biomass enhancement and colonization in the red pepper phyllosphere. Therefore, this study proved that the versatility of *Methylobacterium* as a plant-growth promoting bacteria could be better exploited.

Key Words
Methylotrophy, *Methylobacterium*, ACC deaminase, Auxin Production, Cytokinins.

Introduction

Plant interactions with microorganisms are well-documented phenomena. Symbiotic bacteria that inhabit the rhizosphere and form nodules on the root of legumes are able to assimilate atmospheric nitrogen and provide it to the host plant. Rhizosphere bacteria, including members of the genera *Rhizobium* and *Bradyrhizobium* however, are not the only players involved in plant-microbe symbiosis. Many bacteria are present in the plant phyllosphere and there is evidence that these inhabitants have a significant impact on plant growth and development. Among such inhabitants are the pink-pigmented facultative methylotrophic bacteria (PPFMs), which are members of the Genus *Methylobacterium* and are gram-negative alpha-proteobacteria. These plant associated bacteria are easily detected by their pink color and ability to utilize one carbon compounds, such as methanol, as sole carbon and energy source. They are phylogenetically related to both plant-associated bacteria *Agrobacterium* and *Rhizobium* and have more recently been placed in a clade, which includes a *Methylobacterium* strain that is able to symbrotically nodulate and fix nitrogen in legumes (Sy *et al.* 2001). The quantity of IAA produced and sensitivity of the plant tissue also play important role in several functions, such as root elongation and the formation of lateral and adventitious roots. Cytokinins are N6-substituted adenine derivatives that have diverse effects on important physiological functions in plants and whose level can alter the root functions. PGPB plays a role in reducing ethylene in plants via the action of ACC deaminase (ACCd) enzyme (EC 4.1.99.4) that sequesters and hydrolyzes ACC to α-ketobutyrate and ammonia (Glick *et al*. 1998). It was previously proposed that much of the ACC formed in this reaction are exuded from seeds or plant roots along with other small molecules normally present and may be taken up by the bacteria and subsequently hydrolyzed by the ACCd enzyme. This in turn, would lead to more ACC exudation from inside the plant to maintain the equilibrium thus reducing ACC and the amount of ethylene evolved by the plant (Glick *et al*. 1998). IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACCd activity (Patten and Glick 2002). *Methylobacterium* inoculation increased the IAA concentrations of the plants resulting in increased ACS activity. However, the ACC and ethylene concentrations inside the tissues were reduced due to the activity of bacterial ACCd (Madhaiyan *et al*. 2006). In this present study, we are demonstrating the involvement of ACCd producing *Methylobacterium oryzae* strains isolated from rice in plant growth promotion.

Methods

Bacterial Strains and Culture Conditions

The methylotrophic strains *Methylobacterium oryzae* CBMB20 and *Methylobacterium oryzae* CBMB110 were isolated from rice stem and leaf. The methylobacteria were grown for 72-120 h on ammonium mineral salt (AMS) media supplemented with 0.5% methanol and cycloheximide (30 µg/ml).

In vitro estimation of plant growth promoting traits by methylotrophic strains

Methylobacterium strains grown in AMS media were used for the estimation of IAA and cytokinins. The production of IAA by the methylotrophic isolates was determined according to the method by Bric *et al*. (1991). Sterile supernatants of samples taken at 120 h of growth were analyzed for cytokinin production using immunoassays. To determine ACC deaminase activity, the bacterial isolates were grown in 15 ml of LB broth at 30 ºC until they reached the stationary phase after which, cells were collected by centrifugation (at 8000 g). To induce ACC deaminase activity, the cells were resuspended in 7.5 ml of DF minimal salts medium supplemented with 5 mM of ACC as a sole nitrogen source and then incubated for 40 h at 30 °C with shaking (120 rpm). ACC deaminase activity was determined by measuring the production of α-ketobutyrate and expressed as nM of α-ketobutyrate formed min^{-1} mg $protein^{-1}$ (Penrose and Glick 2003).

Gnotobiotic assays

After surface sterilization, red pepper and tomato seeds were treated with the *Methylobacterium* strains and transferred to growth pouches under aseptic conditions. The culture conditions and the procedure for gnotobiotic growth pouch assay followed that of Penrose and Glick (2003). To check the persistence, treated seeds were transferred to multi-well trays filled with air-dried Wonjo-Mix bed soil and vegetable raising growth medium (Nong-Kyung Co., Ltd, Jincheon-gun, Chungbuk, Republic of Korea) as described by Poonguzhali *et al*. (2008).

IAA and cytokinin in plant extracts

Extracts for IAA and cytokinin assays were prepared by homogenizing the seedlings (1 g) with TBS buffer in a ratio of 1:3 (w/v). Supernatants of the extracts (5000 rpm for 3 min, twice) were used for IAA, trans-zeatin riboside (*t*-ZR), and dihydrozeatin riboside (DHZR) assays. Enzyme-Linked Immunosorbent Assay (ELISA) test kits were used for immunoassays to measure the IAA and cytokinins in the sample. ELISA tests were performed according to kit instructions. The absorbance was read at 405 nm using an ELISA plate reader (BIO-RAD Model 550, Japan).

Results

The *Methylobacterium* strains varied in their ability to utilize ACC and significant differences were observed in the ACC deaminase activity of cell free extracts. CBMB20 and CBMB110 produced 94.5 and 24.7 nmol α-ketobutyrate mg^{-1} of protein h^{-1}, respectively. A quantitative analysis using the Salkowski reagent of the culture liquids of the methylobacteria grown in the defined medium with L-tryptophan and incubated for 5 days produced significantly different amounts of IAA. In the presence of L-tryptophan, the production of IAA by *Methylobacterium* strains CBMB20 and CBMB110 was 2.33 and 4.03 µg/ml, respectively. Immunoassays using ELISA kits were also performed to determine the cytokinins produced by the *Methylobacterium* strains. The cytokinins *t*-ZR, iPA, and DHZR were all present at detectable and replicable levels in the cultures tested, with *t*-ZR present in smaller quantities. The total amount of cytokinins recovered from the cultures varied, but strain CBMB20 produced a significantly higher amount than CBMB110. PPFMs synthesized IAA predominantly by an alternate tryptophan-dependant pathway, through indole-3-pyruvic acid, however, the role of bacterial IAA in plant growth promotion remains undetermined (Ryu *et al*. 2006) (Table 1).

Table 1. ACC deaminase, IAA and cytokinin production of the *M. oryzae* strains isolated from rice

Strains	ACC deaminase activity (nmol α-ketobutyrate mg^{-1} protein h^{-1})	IAA production (µg ml^{-1}culture)		Cytokinin recovered (ng l^{-1}culture)	
		Trp^+	Trp^-	iPA	*t*-ZR
M. oryzae CBMB20	94.48 ± 6.83	2.33 ± 0.11	1.72 ± 0.08	47.01 ± 0.45	32.92 ± 1.43
M. oryzae CBMB110	24.74 ± 4.12	4.03 ± 0.20	1.07 ± 0.05	41.87 ± 1.26	26.23 ± 1.24

The germination percentage and root length of the *Methylobacterium* strain-treated tomato and red pepper seeds were comparatively greater when compared to the uninoculated control. The percentage increase in root length compared to the control was 39.4% when treated with CBMB20, whereas CBMB110 recorded higher increases over the control amounting to 61.3%. These results also matched the results of previous studies with rice and sugarcane crops, where treatment with certain cytokinin-producing *Methylobacterium* strains increased growth (Madhaiyan *et al*. 2005) (Table 2).

Table 2. Effect of *M. oryzae* strains inoculation on the root length of red pepper and tomato under gnotobiotic conditions

Treatment	Root length (cm)	
	Tomato*	Red pepper**
M. oryzae CBMB20	5.81 ± 0.14	8.78 ± 0.17
M. oryzae CBMB110	6.72 ± 0.15	9.78 ± 0.16
Control	4.17 ± 0.12	5.88 ± 0.20

* 15 days old plants; ** 10 days old seedlings.

For the tomato seedlings, no detectable amounts of IAA were found, although the presence of t-ZR and DHZR was recorded. The *Methylobacterium* strains produced significantly higher amounts of t-ZR than the control, with CBMB20 recording the highest, amounting to 0.0125 pmol g^{-1} FW. A similar trend was also seen with DHZR, but the differences were not significant. The effect of *Methylobacterium* inoculation on the plant growth hormones was more prominent in the red pepper seedlings compared to that of the tomato seedlings. The amount of IAA in the treated seedlings significantly differed from that of the control. The cytokinins in the red pepper tissue extract increased with *Methylobacterium* inoculation. The *t*-ZR concentration in the *Methylobacterium*-treated seedlings was significantly increased compared to that of the control and *miaA*$^{-}$ mutant. A similar trend was seen for DHZR, although inversely, CBMB110 produced more at 0.658 pmol g^{-1} FW than CBMB20 at 0.562 pmol g^{-1} FW.

Table 3. Effect of *Methylobacterium oryzae* inoculation on cytokinin concentrations in tomato and red pepper seedlings

Treatment	IAA (pmol g^{-1} FW)	Concentration of Cytokinin (pmol g^{-1} FW)		
		t-ZR	DHZR	Total
Tomato*				
M. oryzae CBMB20	ND	0.0125^{a}	0.475^{ab}	0.4875^{bc}
M. oryzae CBMB110	ND	0.0115^{b}	0.431^{b}	0.4425^{c}
Control	ND	0.0074^{c}	0.468^{ab}	0.4754^{a}
Red pepper**				
M. oryzae CBMB20	61.65^{b}	0.0218^{a}	0.562^{ab}	0.5838^{b}
M. oryzae CBMB110	68.27^{a}	0.0127^{c}	0.658^{a}	0.6707^{a}
Control	60.80^{c}	0.0169^{b}	0.253^{b}	0.2699^{c}

* 15 days old plants; ** 10 days old seedlings; ND – Not determined.

In summary, the total amount of cytokinins in the seedlings greatly varied according to the treatment, with inoculated plants recording significant increases of more than 30% compared to the control (Table 3). *M. oryzae* CBMB20 enhanced the plant biomass and showed phyllosphere colonization using leaf imprinting method in a greenhouse experiment (Figure 1).

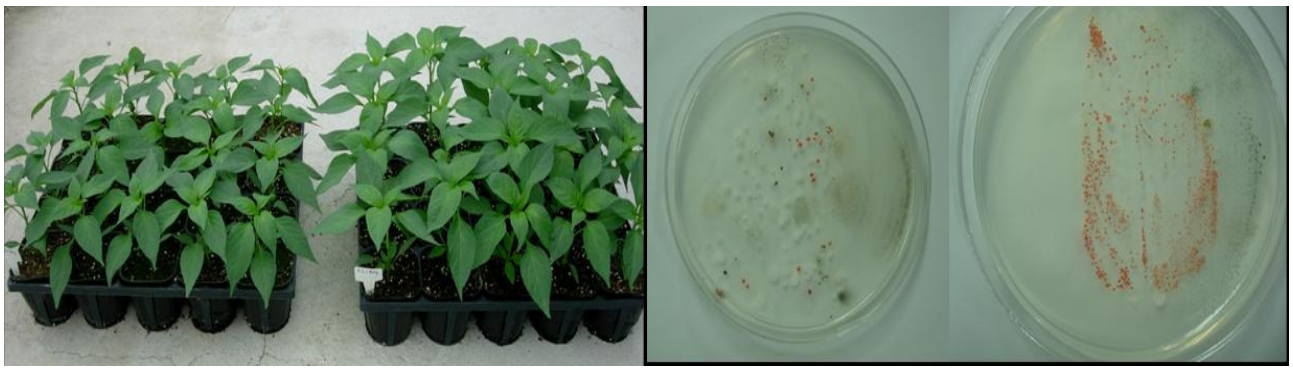

Figure1. Colonization in phyllosphere and plant growth promotion by *M. oryzae* CBMB20 inoculation in red pepper under greenhouse conditions

Conclusion

This present investigation of the inoculation effects of plant-growth promoting methylotrophic bacteria on tomato and red pepper seeds produced satisfactory results, with significant increases in plant growth and plant hormone concentrations over that of the uninoculated control. Therefore, this study proved that the versatility of *Methylobacterium* as plant-growth promoting bacteria could be better exploited.

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PLFA and Enzyme Activities to assess the Impact of Shrub Canopy and Residue Type on Microbial Community during shrub decomposition in semi-arid Senegal

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Abstract
In semi-arid Senegal two shrubs dominate in farmers' fields, *Guiera senegalensis* and *Piliostigma reticulatum*, which are coppiced and burned prior to crop planting. The interactions of these shrubs with the microbial communities are uninvestigated. Our objective was to determine the influence of shrub rhizosphere and residue chemistry on the microbial community during the decomposition process. The experimental design was a 2X3 factorial design with two soil treatments (beneath and outside the influence of the shrub) and three residue amendments (leaf, leaf+stem and control). The samples were incubated in laboratory conditions with destructive samplings at days 7, 14, 45, 75, or 105 after incubation. Shrub rhizosphere on the microbial communities was stronger than the residue type effect. The fungal biomarkers were more closely correlated than other microbial groups to residue chemistry. Furthermore, Gram-positive bacteria and the fungal markers 18:2ω6c and 18:1ω9c were highly correlated with both cellulase and β-glucosidase activities. This study showed PLFA profiling of microbial communities was sensitive to temporal dynamics and residue amendments during residue decomposition and that correlation of PLFA markers with hydrolytic enzyme activities provides a means of inferring the functional role of microbial groups that dominate over time during decomposition.

Key Words
PLFA, Fungi markers, enzymes, rhizosphere, shrub litter.

Introduction
Shrubs and trees in semi-arid environments are known as islands of fertility where soil beneath the canopy is characterized by a high C and N content, as well as high microbial biomass and activity (Gallardo and Schlesinger 1995). In the semi-arid Senegal, two native and dominant shrubs, *Piliostigma reticulatum* and *Guiera senegalensis* are coppiced and burned in the spring to prepare for the next cropping period. Relatively little is known about *P. reticulatum* and *G. senegalensis* as islands of fertility and their role in decomposition. Nor has there been an attempt to develop non-thermal management systems to optimize the use of the litter for improving soil quality and crop productivity. Fundamental studies examining the shrub canopy influence on the microbial community structure and decomposition are needed. The objectives of this research were to study the structure and activity of the microbial communities during litter decomposition for *G. senegalensis* and *P. reticulatum* with respect to 1) impacts of soils from beneath or outside the shrub canopies and 2) shrub residue chemistry using PLFA and enzymes activities methods.

Materials and methods
Site Description and Laboratory Incubation Study
The experimental location was the semiarid agro-ecological zone in the semi-arid Senegal characterized by a tropical sudanian climate with potential evapotranspiration of 1800mm/yr. A Dior loamy sand was collected for *G. senegalensis* near Bambey (precipitation of 400-600 mm/yr) and similarly another set of sandy loam soil samples was collected for *P. reticulatum* near Kaolack (precipitation of 700-1000 mm/yr). Soils are low in C and N. The incubation study had a completely randomized 2 x 3 factorial design for each shrub type with two soil treatments (0-5 cm depth soil beneath or outside the shrub canopy) and three residue treatments (leaf, 60% stem plus 40% leaf, and control soil with no residue). Residues were mixed with 100 g of soil and placed in a 0.25 L plastic cup and incubated at 25°C (0.7% w/w). Soils were sampled destructively at days 7, 15, 45, 75 and 105 of the incubation period.

Phospholipids fatty acids analysis
Microbial community structure was determined by analysis of PLFA using a modified method described by Bligh and Dyer (1959). Fatty acids were extracted in three steps from 3g of triplicate sub-samples soil with a one-phase chloroform-methanol- phosphate buffer solvent. The polar lipid fraction was trans-esterified with mild alkali to recover the PLFA as methyl esters in 300 µl of hexane. PLFA were analyzed by gas chromatography (GC) (temperature ramping 120°C to 260°C at a rate of 5ºC per min). Total PLFA ($PLFA_{tot}$) was summed across each sampling date and was used as an indicator of microbial biomass.

Enzyme activities
Activities of two enzymes related to the C cycle (β-glucosidase and cellulase) were measured. Cellulase were determined by incubating 1 g of soil with 10 mL of 2 M acetate buffer (pH 5.5) containing the substrates, carboxymethyl cellulose sodium salt (0.7% w/v) (Schinner and von Mersi 1990). The β-glucosidase activity was determined by measuring the product *para*-nitrophenol (*p*NP) after incubation of fresh soil in the presence of the substrate, pNP-glucopyranoside as described by Tabatabai (1994).

Statistical Analysis
Effects of residue amendment and shrub canopy on microbial PLFA groups were analyzed using SAS. Shifts in PLFA profiles over time were analyzed by non-metric multidimensional scaling (NMS) using the PC-ORD. To assess the difference in community PLFA profiles according to location, substrate amendment and time of incubation, permutational multivariate analysis of variance (PerMANOVA) was performed. Amount of PLFA have been also correlated with enzyme activities as well as some specific PLFA groups using S-plus.

Results
Guiera senegalensis
There was a residue type effect for all communities groups ($p < 0.03$) except for the bacterial community PLFA at day15 with soil beneath the canopy having higher PLFA levels, regardless of the residue amended. A share of 95% of the data was explained by the first two axes using NMS analysis. The fungal marker 18:2ω6c was highly correlated with axis one ($r = 0.94$) whereas the actinomycete marker 10Me 16:0 was negatively correlated with axis one ($r = -0.91$). Correlation among microbial PLFA, showed that the fungal to bacterial ratio was highly correlated with both axis 1 ($r = 0.85$) and axis 2 ($r = 0.79$). A significant difference was found between control and amended soils ($p < 0.001$) (Fig. 1). However, the difference between soils amended with leaf vs. leaf/stem mix was insignificant. There was a strong difference between soil taken beneath shrubs and outside shrubs for the control soil ($p < 0.001$) and within each sampling date for amended soil ($p < 0.01$).

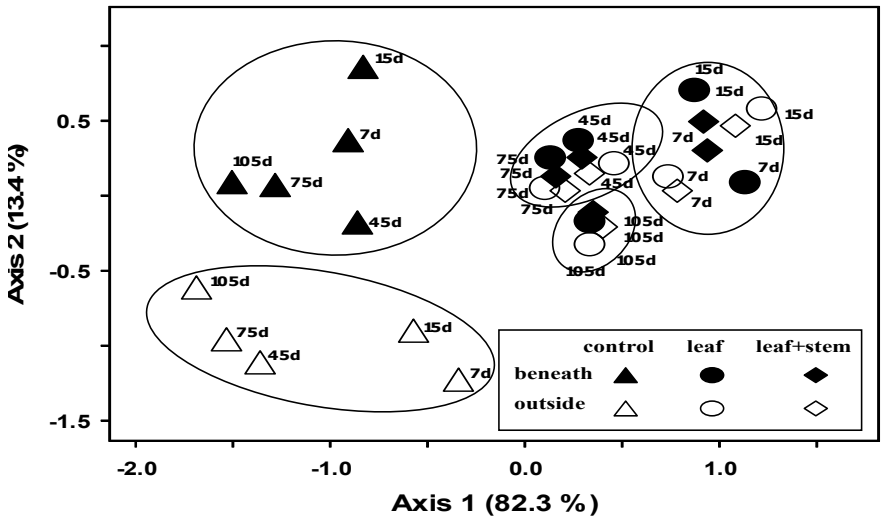

Figure 1. NMS representation of soil sample distances based on the mol % of 30 PLFA peaks extracted from soil associated with *Guiera senegalensis* amended with different residues and incubated over time (d = days of

Piliostigma reticulatum

The residue effect was significant for all samples ($p < 0.03$) with higher PLFA amount with soil amended with leaves (Table 1). $PLFA_{tot}$, bacterial, fungal and actinomycetal PLFA were higher beneath than outside canopy up to day 45. The location effect as well as the time effect was strong for all samples ($p < 0.001$). The marker 18:0 2OH and the fungal markers 18:2ω6c and 18:1ω9c had the highest positive correlation with axis 1 respectively ($r = 0.78$, $r = 0.77$ and $r = 0.76$). FUN/BACT ratio was strongly correlated with axis 1 ($r = 0.86$) whereas Gram-negative bacteria and the fungal group were highly correlated with axis 2 respectively. There were a strong difference between control soil and amended soil ($p<0.0001$) and a stronger difference between soil beneath canopy and outside canopy with respect to sampling date ($p<0.001$).

Table 1. PLFA (nmol g^{-1} soil) for soil taken beneath and outside canopy of *P. reticulatum* (n=3).

	PLFA averaged across all sampling date				
		Beneath canopy		Outside canopy	
Residue	Taxonomic groups				
Control	10Me 16:0	7.3	(0.9)	5.3	(0.4)
	18:2ω6,9c	2.3	(0.5)	1.1	(0.5)
	FUN/BACT	2.0	(0.1)	1.6	(0.2)
Leaf	10Me 16:0	23	(2.1)	15	(2.7)
	18:2ω6,9c	34	(3.0)	19	(1.1)
	FUN/BACT	4.3	(0.3)	4.2	(1.0)
Leaf+stem	10Me 16:0	21	(1.1)	13	(1.1)
	18:2ω6,9c	28	(4.0)	17	(1.0)
	FUN/BACT	4.1	(0.5)	4.1	(0.8)

Correlation of PLFA with Enzyme Activities

For *G. senegalensis* the PLFA markers 17:0a, 15:0 and 15:0i had the strongest correlation with β-glucosidase with respectively ($r = 0.68$) for the first two markers and ($r = 0.62$) for 15:0i. The fungal marker 18:2ω6c had a correlation of $r = 0.6$ with β-glucosidase. The marker 10Me 16:0 had the weakest correlation ($r = 0.25$). Cellulase activity was more correlated with 17:0a ($r = 0.70$) and 15:0 ($r = 0.75$). For *P. reticulatum* the fungal marker 18:1ω9c was highly correlated with both β-glucosidase ($r = 0.72$) and cellulase activity ($r = 0.84$). The fungal marker 18:2ω6c also had a higher correlation with both enzymes $r = 0.6$ for β-glucosidase, and 0.8 with the cellulase activity. The marker 10Me 16:0 is the least correlated with the β-glucosidase activity ($r = 0.18$) and also its correlation is low with cellulase ($r = 0.25$).

Discussion

Amendment with leaves had a moderately higher amount of microbial PLFA than did soils amended with a mixture of leaves and stems. Substrate chemistry may strongly influence the composition of the decomposer communities which in turn may affect decomposition of plant material (Heal *et al*. 1997). This is particularly true for the fungi where fungal PLFA were significantly higher in soils amended with leaf than soils amended with leaf + stem. In this experiment, the amount of $PLFA_{tot}$ depended primarily on the time of incubation; for the fungi group it depended primarily on the residue type. This shows again the ubiquity of the fungi to respond readily to substrate availability and in relation to the chemistry of the residues added to soils. This was also shown by Broder and Wagner (1988) who reported fungal response to residue chemistry during successional stages of wheat straw decomposition. The highest correlation of the fungal markers (18:2ω6c, 18:1ω9c) with axis 1 may be due to the fact that fungi communities responded readily to the available C sources at early stages of decomposition and then decreased thereafter. The important role of fungi in the C cycle was shown by the generally high correlations of cellulase and β-glucosidase activity with the fungal markers 18:2ω6c and 18:1ω9c for both shrubs species. Our results are consistent with Schutter and Dick (2002) who showed that fungal markers, 18:2ω6c and 18:1ω9c were stimulated by the addition of cellulose to soils.

Gram-positive bacteria markers also had a strong correlation with cellulase and β-glucosidase activities in amended soils. Unlike Gram-negative bacteria which colonize readily decomposable compounds Gram-positive bacteria can thrive on more recalcitrant materials and under more stressed environments and, furthermore can dominate in agricultural soils that typically are under more stress (Haack *et al*. 1994).

Our results were consistent with other studies that showed the influence of soil organic matter content, vegetation type and soil management on soil microbial composition (Schutter and Dick 2002; Jackson *et al*. 2003; Jandl *et al*. 2005).

Conclusion

The dominant factor that resulted in the largest shift in microbial communities is the effect of shrub canopies/rhizosphere over non-rhizosphere soil. Correlation analysis provided indirect evidence that residue chemistry affected soil communities with fungi being the most responsive to type of litter added than any other functional groups. Strong correlations of cellulase and β-glucosidase activities with fungal PLFA provided evidence for the dominance and ability of fungi to degrade the two shrub species residues. Gram-positive bacteria had high correlations with enzyme activities and it was particularly stimulated by *G. senegalensis*-amended soils. These results clearly show that microbial communities beneath shrubs are more important and distinctly different than soils outside the influence of the two shrub species. These results have practical implications in that it appears that the presence of shrubs did enhance the decomposition process. This is a reasonable basis to begin developing non-thermal residue management with a goal of replacing the current destructive farming practice of burning residue.

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Preliminary Study on Phosphate Solubilization and K-releasing Abilities of *Rhizobium tropici* Martinez-Romero *et al.* Strains from Woody Legumes

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Abstract
Experiments were conducted on phosphate solubilization and K-releasing abilities of standard strain CGMCC 1.2540^T of *Rhizobium tropici* Martinez-Romero *et al*. (1991) and 22 strains of *Rhizobium tropici* Martinez-Romero *et al*. (1991) which were separated from woody legume plants in the Jianfengling Nature Reserve, Hainan Province. None of the tested strains have shown the ability to solubilize organic phosphate on Mongina organic culture medium, but have demonstrated K-releasing ability on K-releasing culture medium. 20 strains have the ability to solubilize inorganic phosphate of $Ca_3(PO_4)_2$. Different strains indicate great differences in phosphate solubilization and K-releasing abilities, particularly so in phosphate solubilization. The weakest ability of phosphate solubilization by the strain of Caf438 and the strongest by Caf341 increased the available phosphoru in the medium by 0.796 mg L^{-1} and 628.57 mg L^{-1} respectively, of which the difference is 788 times; The abilities of phosphate solubilization and K-releasing are irrelevant of hosts. The strains of *Rhizobium tropici* Martinez-Romero *et al*. (1991) on the same host show apparent differences on phosphate solubilization and K-releasing abilities. The strains of Caf336, Caf344 and Caf414 are provided with more powerful phosphate solubilization and K-releasing abilities.

Key Words
Woody legume, rhizobia, phosphate-solubilized halo, phosphate solubilization, content of available P, K-releasing ability, content of available K

Introduction

The symbiosis of rhizobia and legume plants is the strongest system in bio-fixation of nitrogen, of which the nitrogen fixed accounts for 65% of all the nitrogen fixed biologically (Danming *et al.* 2002; Zabran, 1999; Denarie and Roche 1991). Moreover, the developed root system of woody legumes make them stronger in nitrogen fixation in comparison with grain legumes of soybean, peanuts, broad bean and others (80-210 kgN/ ha.a), which is 43-581 kgN / ha.a (Fuli and Zhengjia 2000). The current studies on rhizobia are concentrated on the function of nitrogen fixation and their relationship with host plants, while the abilities of their phosphate solubilization and K-releasing are less dealt with. N, P and K are three main essential nutrition elements for plants. In China, the total content of P in soil is high, but of which 95% is not available in the forms of aluminnosilicate, phosphorite, etc. that is difficult to be used directly by plants. Soil is a natural reserve of potassium, the K content of the tilled soil layer is about 26,100 kg/hm^2, of which 90% exists in potassium feldspar, mica and other silicate minerals. Those are stable minerals that could not be used directly by plants, leading to the phenomenon of K rich but available K lacking in soil. Phosphate solubilizing microbe convert and make available P in soil and the study of P solubilization by bacteria mainly deals with silicate bacteria (Baogui and Bin 2005; Jian *et al.* 2000; Fengding *et al.* 1997). But those microbes are found in a free state in soil, which hinders the conversion and use of unavailable P and K in soil, owing to the competition with indigenous microbes and their survival time. P solubilization and K releasing by rhizobia is seldom reported. Rhizobium and its symbiotic plant forms a specific structure – root nodule, which is endowed with a unique advantage in survival time and competibility compared with other bacteria. The screening of P solubilizing and K-releasing rhizobia strains has high academic, ecological and economic values.

Material and Methods

Bacterial strains

The 22 strains of Rhizobium tropici Martinez-Romero *et al.* (1991) were isolated from the root nodules of legume plants in the Jianfengling Nature Reserve, Hainan Province. The strains of Caf224, Caf225, Caf226 were isolated from Ormosia balansae Drake, Caf276, Caf278 and Caf279 were isolated from Desmodium triquetrum (Linn.) DC., Caf333, Caf334, Caf336 were isolated from Desmodium heterocarpon (L.) DC., Caf341, Caf344 were isolated from Indigofera suffruticosa Mill. Caf414, Caf415, Caf416 were isolated from Ormosia semicastrata Hance f. Litchifolia, and Caf436, Caf437, Caf438, Caf439, Caf440, Caf443, Caf444, and Caf446 were isolated from Acacia mangium Willd., while standard strain CGMCC 1.2540T was from cgmcc.

Media and methods

1. Bevel culture media: Rhizobia culture media-1 (Yuguang 2007).
2. Selecting strains of inorganic phosphate-solubilized on PKO medium and solubilize organic P (lecithin) on Mongina organic culture medium. Checking up the phosphate-solubilized halo of the tested strains
3. Liquid culture medium (Chuanjin and Lin 2002).
4. The inorganic phosphate culture medium：Glucose 10 g, $(NH_4)_2 SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, NaCl 0.3 g, KCl 0.3 g, $FeSO_4 \cdot 7H_2O$ 0.03 g, $MnSO_4 \cdot 7H_2O$ 0.03 g, $Ca_3(PO_4)$ 2 5 g, distilled water 1000 mL, pH 7.2, 110 ^oC Sterilization for 30 min.
5. The Organic phosphate culture medium: Glucose 10 g, $(NH_4)_2 SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, NaCl 0.3 g, KCl 0.3 g, $FeSO_4 \cdot 7H_2O$ 0.03 g, $MnSO_4 \cdot 7H_2O$ 0.03g, lecithin 2 g, $CaCO_3$ 5 g, distilled water 1000 mL, pH 7.2, 110 ^oC , Sterilization for 30 min.
6. Determining available P content of the culture solution used Mo anti-antimony colorimetry.
7. Ashby culture medium (Yuguang 2007)
8. KH_2PO_4 0.2 g, $MgSO_4 7H_2O$ 0.2 g, NaCl 0.2 g, $CaCO_3$ 5.0 g mannite 10 g, $CaSO_4 2H_2O$ 0.1 g, distilled water 1.0L, pH7.0. Choose the colony with slime.
9. K-releasing culture medium (Institute of Soil Science 1985)
10. Saccharose 5.0 g, Na_2HPO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.5g $FeCl_3$ 0.005 g, $CaCO_3$ 0.1 g, $KAlSi_3O_8$ 5 g, distilled water1.0 L. Determine available P content of the culture solution with Atomic Absorption Spectrophotometer Method.

Data processing

Data processing using Excel and Spss16.0

Result

Selecting the rhizobia strains of phosphate solubilization

Checking the phosphate-solubilized halo.On PKO inorganic medium flat, 20 strains have generated phosphate-solubilized halo except Caf226, Caf438 and CGMCC 1.2540T. The result shows that the 20 strains could solubilize nullification P ($Ca_3(PO_4)_2$). On Mongina organic culture medium, all of 23 tested strains show no phosphate-solubilized halo. Figure1 shows the phosphate-solubilized halo of Caf341 strain On PKO inorganic medium flat.

Figure 1. Phosphate-solubilized halo of Caf341 strain.

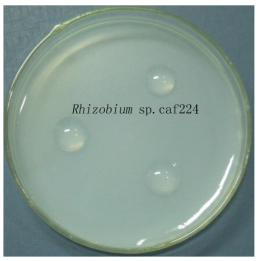

Figure 2. Caf224 colony on potassium medium culture.

The ability of diferent Rhizobia isolated at solubilizing inorganic phosphate

Table 1. The Ability of Diferent Rhizobia Isolated at Solubilizing Inorganic Phosphate.

Strains	Content of available P of the *Rhizobia* culture with $Ca_3(PO_4)_2$ as only P source	Strains	Content of available P of the *Rhizobia* culture with $Ca_3(PO_4)_2$ as only P source
Caf224	13.81**	Caf415	14.44**
Caf225	22.18**	Caf416	45.20**
Caf226	2.51	Caf436	585.06**
Caf276	528.56	Caf437	418.69**
Caf278	421.85	Caf438	1.86
Caf279	101.69**	Caf439	139.36**
Caf333	239.79**	Caf440	170.75**
Caf334	618.40**	Caf443	264.90**
Caf336	403.01**	Caf444	499.31**
Caf341	629.63**	Caf446	126.80**
Caf344	426.87**	CGMCC1.2 540T	1.89
Caf414	422.35**	ck	1.06

**level0.011

In Table 1, content of available P of 20 strains with phosphate-solubilized halo is higher than that of the control evidently. Different strains indicate great differences in phosphate solubilization. The weakest ability of phosphate solubilization by the strain of Caf438 and the strongest by Caf341 increased the available phosphate in the medium by 0.796 mg L^{-1} and 628.57 mg L^{-1} respectively, of which the difference is as great as over 788 times; The contents of available P in culture liquid of Caf341, Caf334, Caf436, Caf276, Caf444, Caf344, Caf414, Caf278, Caf437 and Caf336 are more than 400 mg L^{-1}.All of 23 strains are Rhizobium tropici Martinez-Romero *et al*. (1991) The result shows that different strains of same species have different abilities of phosphate solubilization.

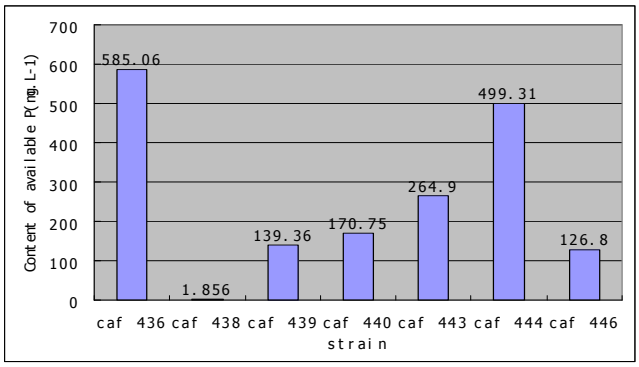

Figure3. Contents of available P of the *Rhizobia* culture (host: *Acacia mangium* Willd.)

The abilities of phosphate solubilization of strains which were isolated from the same host are different. Caf436 and Caf438 were isolated from the root nodule of *Acacia mangium Willd*, which have increased the available phosphate in the culture liquid by 0.796 mg L^{-1} and 584 mg L^{-1} respectively, of which the difference is as great as over 733 times.

Selection of strains with K-releasing ability

All the 23 tested strains could grow on Ashby culture medium and K-releasing culture medium. All of the colonies are smooth, transparent and with slime. This shows that all strains could utilize K of $KAlSi_3O_8$. See Figure 2.

Determination of K-releasing ability

The content of available potassium from the filtrates of culture were determined with Atomic Absorption Spectrophotometer Method (Table 2). All of the tested strains were apparently different in K-releasing ability from the control (F0.01=0.000<0.01), with a sharp contrast between Caf224 and Caf416 against the standard strain of CGMCC1.2540. Caf224, Caf416, Caf420, Caf278, Caf440, Caf341, Caf344, Caf414 and Caf415 of Rhizobium tropici Martinez-Romero *et al*. (1991) show apparent differences in on phosphate solubilization and K-releasing abilities, compared with the standard strain. And the ability of strains in K-releasing is not related with hosts.

Table 2. Available Potassium Contents in Different Rhizobia Cultures.

	Available potassium content （mg/L）					Available potassium content （mg/L）			
Strains	1	2	3	Average	Strains	1	2	3	Average
Caf224	8.272	7.735	8.248	8.272**	Caf415	7.434	7.634	7.908	7.659**
Caf225	7.256	6.296	6.729	7.256**	Caf416	8.39	7.998	7.692	8.027**
Caf226	7.706	7.241	7.882	7.610**	Caf436	6.422	6.849	6.452	6.574**
Caf276	6.769	6.293	7.315	6.792**	Caf437	6.849	7.185	6.786	6.940**
Caf278	6.964	8.211	6.704	7.293**	Caf438	7.288	7.066	6.668	7.007**
Caf279	6.977	6.804	6.720	6.834**	Caf439	7.034	6.855	6.609	6.833**
Caf333	6.964	7.750	6.789	7.168**	Caf440	7.24	7.537	7.21	7.329**
Caf334	7.111	6.886	7.495	7.164**	Caf443	6.693	7.806	6.709	7.069**
Caf336	7.794	7.512	7.446	7.584**	Caf444	6.840	6.701	6.673	6.738**
Caf341	7.249	7.834	7.091	7.391**	Caf446	6.764	6.450	7.969	7.061**
Caf344	7.763	7.534	7.153	7.483**	CGMCC1.2540^T	6.783	8.285	6.606	7.225**
Caf414	8.277	7.762	7.535	7.858**	Check	4.791	5.263	5.470	5.175

**level0.01

The correlation of abilities on phosphate solubilization and potassium releasing

Table 3. The Correlation of Abilities on P Solubilization and K Releasing.

		Content of available K	Content of available P
VAR00002	Pearson Correlation	1	-.007
	Sig. (2-tailed)		.954
	N	72	72
VAR00003	Pearson Correlation	-.007	1
	Sig. (2-tailed)	.954	
	N	72	72

It is seen from Table 3, the abilities of strains on phosphate solubilization and K–releasing are irrelevant in between. The result shows that phosphate solubilization and K–releasing are effected by different mechanisms.

Results and Discussion

1 The 22 strains which were separated from woody legumes in the Jianfengling Nature Reserve, Hainan Province have the ability to release K, and of which 20 have the ability to solubilize inorganic phosphate of $Ca_3(PO_4)_2$. The strains with strong P solubilization ability are Caf341, Caf334, Caf436, Caf276, Caf444, Caf344, Caf414, Caf278, Caf437 and Caf336.

2 Different strains demonstrate great differences in the abilities to convert P and K, particularly so in the conversion of P. The weakest ability of phosphate solubilization by the strain of Caf438 and the strongest by Caf341 increased the available phosphate in the medium by 0.796 mg L^{-1} and 628.57 mg L^{-1} respectively, of which the difference is as great as over 788 times.

3 The abilities of phosphate solubilization and K-releasing are irrelevant of hosts. The strains of Caf436, Caf437, Caf438, Caf439, Caf440, Caf443, Caf444 and Caf446 were all on the same host of *Acacia mangium Willd*, which, however, present a sharp contrast in between in the contents of available P and fast K.

4 Different strains of the same species indicate great differences in phosphate solubilization and K-releasing abilities. All the strains tested are of *Rhizobium tropici* Martinez-Romero *et al*. (1991) but different strains show a great contrast of available P and fast K in the culture.

5 The abilities of rhizobia in P solubilization and K–releasing are not correlated, which indicates different mechanisms for P solubilization and K–releasing, awaiting further study.

6 None of the rhizobia of 26 strains tested in the experiment is able to solubilize the lecithin of organic phosphate. Whether the rhizobia can solubilize other organic phosphates calls for further experiment.

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Role of phosphate solubilising bacteria on availability phosphorus in Oxisols and tracing of phosphate in corn by using ^{32}P

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Abstract
Phosphorus deficiency is one of the most growth-limiting factors in acid soils in the tropics. Phosphorus fixation results in low P use efficiency in acid soils. Using isotopes as tracers, the percentage of utilization by plants of the P derived from either organic or inorganic fertilizer can be determined. This investigation was conducted with soybean biomass and corn biomass treatments, phosphate-solubilising bacteria (PSB), and rock phosphate (RP). ^{32}P-free carrier was applied to thirty six pots and another set of thirty six pots which were identical with the first in all aspects but without ^{32}P labeling. Corn was grown on a Typic kandiudox from Depok, West Java, Indonesia for 8 weeks. The radioisotope technique with $KH_2{}^{32}PO_4$ carrier free was used to trace the distribution of P in shoot of corn, to calculate the P-fertilizer use efficiency and also to detect phosphate uptake by corn. The ^{32}P activity in corn was measured by Liquid Scintillation Counter. The result of this study showed that application of PSB in biomass significantly increased available-P in an Oxisol. Uptake of P by corn from biomass or RP was higher than from PSB activity. In addition, P-fertilizer use efficiency of corn was very low and less than 5%.

Key Words
Acid soil, *Bacillus*, phosphate-solubilising bacteria, radiotracer ^{32}P, rock phosphate.

Introduction
The term available-P is often used to describe the amount of soil P that can be extracted from solution or taken up by plant roots and utilized by the plant to growth and develop during its life cycle. The concentration of available-P is always low because of continuous plant uptake. Phosphorus fertilizer efficiency in acid soils is less than 20% due to P fixation through P precipitation by soluble Fe and Al, and adsorption by Fe oxides. Phosphorus sorption may decrease with pH increase in acid soils that is caused by precipitation of amorphous Fe and Al oxides, a greater competition of OH^- with phosphate ions for sorption and an increase of negative charges on soil particles. Organic anions with low molecular mass may coordinate with soluble Fe or Al to make some complexes more stable than Fe or Al phosphates that prevent formation of Fe or Al phosphates (Srivastava *et al.* 2007), and also with allophane minerals (Violante and Gianfreda 2000). Phosphate-solubilising bacteria (PSB) inoculants have been assayed but their effectiveness in the soil-plant system is still unclear. In addition, the role of the inoculated PSB that supplies P to the plant seems limited because the transient nature of the compounds released by PSB responsible for phosphate solubilization, and because the possible re-fixation of phosphate ions on their way to the root surface, if any solubilization does take place (Barea *et al*. 2007). Many researchers prove that PSB plays a key role in soil organic P (P*o*) transformations (Frossard *et al.* 1995) through excretion of phosphatase enzymes (Eichler *et al.* 2004), mineralization of P from organic sources (Gressel and McColl 1997), and also synthesis and release of P*o* (Oberson *et al.* 2001). In addition, microorganisms can solubilize sparingly soluble P*i* forms (Iyamuremye *et al*. 1996). Isotopic dilution method is one of the methods used to evaluate of the agronomic effectiveness of P fertilizers. In the isotopic dilution method, which is realized with or without carrier, the isotope must be applied in the same chemical and physical form as the element to be determined. The objectives of this study were to determine the effect of phosphate-solubilising bacteria on available-P in an Oxisol and to tracer of phosphate on corn.

Methods
A Typic kandiudox from Depok, West Java was used in this study. The soil used for this study has the average top soil depth of 0.10 - 0.25 m with the following characteristics: pH (H_2O) 4.3; 1.82% organic C; 0.19% total N; 35 mg/kg available P, cation exchange capacity 14.16 $cmol_{(+)}$/kg; 0.09 K^+, 2.57 Ca^{2+} and 0.99 Mg^{2+} $cmol_{(+)}$/kg. *Bacillus sp.* Phosphate-solubilising bacteria in corn and soybean biomass and rock phosphate were used as treatments. The chemical composition of soybean biomass and corn biomass used as bacteria carriers were 31.53% and 27.5% organic C; 1.76 % and 0.67% total N; 3.45 % and 10.27% sucrose; 11.90 % and 10.28 %

glucose; 6.32% and 18.55 % cellulose. Treatments applied for this experiment were as follows:

1. no application of solubilising bacteria and rock phosphate (as control treatment).
2. application of soybean biomass with no addition of rock phosphate,
3. application of phosphate-solubilising bacteria in soybean biomass with no addition of rock phosphate,
4. application of corn biomass with no addition of rock phosphate,
5. application of phosphate-solubilising bacteria in corn biomass with no addition of rock phosphate,
6. direct inoculation of phosphate-solubilising bacteria into soil with no addition of rock phosphate,
7. application of rock phosphate only,
8. application of soybean biomass with addition of rock phosphate
9. application of phosphate-solubilising bacteria in soybean biomass with addition of rock phosphate,
10. application of corn biomass with addition of rock phosphate
11. application of phosphate-solubilising bacteria in corn biomass with addition of rock phosphate,
12. direct inoculation of phosphate-solubilising bacteria into soil with addition of rock phosphate,

Each treatment was mixed with 7 kg of soil and placed in a plastic pot. Approximately 80g of each soybean and corn biomass containing $\pm 10^{10}$ CFU of *Bacillus sp* per gram and 4.5 g of rock phosphate were applied accordingly. The twelve treatments were arranged in a completely randomized design with three replicates than constituting thirty six pots. A second set of thirty six pots that were identical with the first set in all aspects but without ^{32}P labeling, were maintained to study the effect of the treatments on the corn growth. The radiotracer ^{32}P applied was from a stock solution of ^{32}P -free carrier of which 20 mL was added to each of the first set of thirty six pots to give an activity of 52.36 MBq/pot.

Three pre-germinated seeds of corn cultivar Arjuna from Indonesia were planted in each pot and grown in a glasshouse. After 8 weeks, corn shoots were harvested by cutting 5 cm above the soil surface. Dry matters, ^{32}P, P content and total P-uptake of plant materials were then analyzed. The ^{32}P activity was counted by the Carenkov method using a liquid scintillation counter carried out at the Centre for Research and Development of Isotopes and Radiation Technology, National Nuclear Energy Agency of Indonesia. From the radio assay data, the % P derived from fertilizer (Pdff), % P derived from soil (Pdfs), and P use efficiency (PUE) were computed. Soil samples from the second set of thirty six pots were analyzed for available P (Bray I) and pH (1:5).

Results and discussion

Effect of treatments on soil pH and available-P

The pH value for all treatments increased if compared with control (Figure 1a). The effect of phosphate-solubilising bacteria in biomass carrier on pH was likely associated with the production of OH^- ions by ligand exchange mechanisms that occurred between organic acids and hydroxyl Fe and Al in soils (Iyamuremye *et al*. 1996). Phosphate-solubilising bacteria in soybean biomass with addition of RP increased available-P higher than that in corn biomass and that without solid carriers (Figure 1b). This seems to be related to the different composition of the biomass. The soybean biomass has N content higher that the corn biomass but has lower C/N and lignin ratios than the corn biomass. Residue factors include chemical composition, C/N ratio, lignin content, and the size of residue particles (Johnson *et al.* 2007). Residue C/N ratio is a common indicator of residue quality but is not necessarily an accurate predictor of decomposition rate (Handayanto *et al.* 1994). Available-P of all treatment increased around 5.34% -

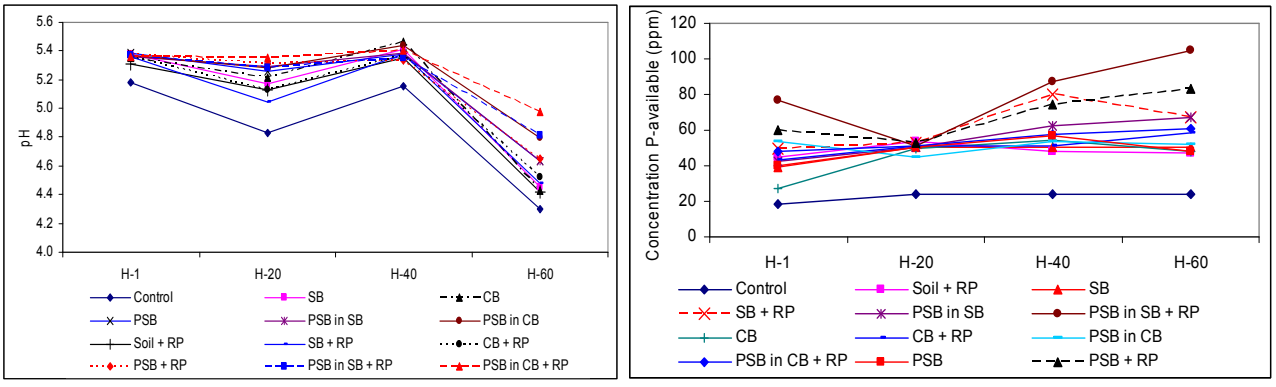

Figure 1. Concentration of soil pH (a) and soil available-P (b) affected by appllication of phosphate-solubilising bacteria (PSB) in biomass with and without RP. SB = soybean biomass; CB = corn biomass; RP= rock phosphate.

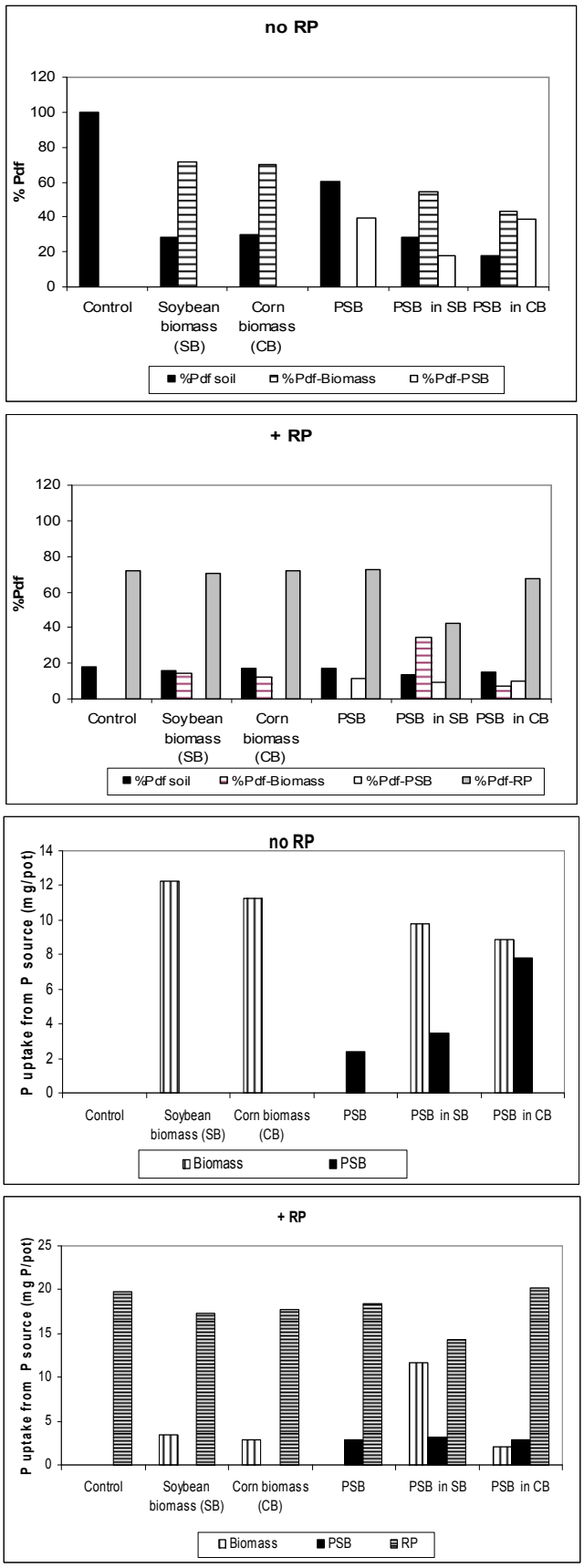

Figure 2. The %Pdff, uptake of P by corn and P use efficiency (PUE) affected by appllication of phosphate-solubilising bacteria (PSB) in biomass with and without RP.

76.71% at 1 to 60 days after planting. The mechanisms were seemed to be the followings: (i) decrease of soil pH because of solubility of Ca-P form; (ii) competition of organic anion with orthophosphate anion due to sorption sites; (iii) ligand exchange; (iv) organic P mineralization. There were no dominant mechanisms

since correlation coefficients between available P with other variables (P-organic, P-inorganic and pH) are less than 0.5.

The %Pdff, uptake of P by corn and P use efficiency (PUE)
The PSB activity in both of biomass had a contribution in shoot phosphorus content of around 18.11% - 39.39%. Addition RP gave different model on P uptake by plant which was more uptake from RP than other source (Figure 2a and 2b). Apllication of PSB in CB without RP provided highest phosphate concentration on corn shoot about 16.64 mgP, there were 7.80 mg P from PSB activity, 8.80 mg P from RP and 3.69 mg P from soil. On the contrary, activity PSB in SB offer only 3.42 mg P. Effect of RP addition on concentration of phosphate uptake was significant, there was about 14.26 – 20.14 mg P/pot or 48.87 – 80.14% total concentration of P uptake by plant. The P-use efficiency (PUE) in corn ranged from 2.12 – 2.78% (biomass), while addition of RP about 1.39 – 3.53%. The PUE of corn was similar to that reported before (Hakim 2002), but was lower than the value of Mohanty *et al.* (2006).

Conclusion
This study showed that application of phosphate-solubilising bacteria in biomass significantly increased available-P in an Oxisol. Uptake of P by corn from biomass or RP was higher than from PSB activity. In addition, P-fertilizer use efficiency of corn was very low and less than 5%. This means that around 95%-97% of the P-fertilizer applied was still in the soil, and was expected to give a residual effect to next crops.

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Root contributions to long-term storage of soil organic carbon: theories, mechanisms and gaps

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Abstract
The depth to which plants locate their roots has important but yet poorly understood implications with regard to the profile distribution and dynamics of soil organic carbon (SOC). We compared the profile distribution of fine root biomass (FRB) with depth distribution of SOC, based on data recalculated from published literature. Mechanisms through which roots might contribute to long-term storage of SOC were reviewed. There was general agreement across previous studies that over 60% of SOC were in the top 0.3 m of soil, where FRB was concentrated. However, studies in which depth distribution of SOC was simultaneously compared to profile distribution of RB were not readily available, suggesting that this area of research has received limited attention. There is a paucity of empirical evidence to lend support to theorised mechanisms through which roots stabilise SOC. The relationship between profile distribution of roots and depth distribution of SOC must be evaluated on-site for defined landuses. A standardised format for presenting results must be developed and agreed upon to ease interpretation of the results. National Soil Science Societies may have a significant role in this process and this 19th World Congress of Soil Science will be an opportune assembly for dialogue.

Key Words
Carbon sequestration, climate change, landuse systems, rooting depth, soil aggregate stability.

Introduction
Plant roots differ in their capacity to explore the soil volume depending on the geographical location, botanical composition of the vegetation, and prevailing environmental factors (Canadell *et al.* 1996; Casper and Jackson 1997; Schenk and Jackson 2002). The relative importance of these differences and their implications for SOC sequestration belowground, are a subject of interest. It is feared that climate change could induce sweeping changes in the botanical composition of plant communities, especially where differences in rooting among plants already exist (Schenk and Jackson 2002). The impact of these changes on the storage and dynamics of C belowground are not well understood. We hypothesise that if roots are an important SOC input and also contribute to SOC stabilisation, then the depth distribution of SOC should follow the same pattern as that of roots. This paper reviewed existing literatures on FRB and SOC, and discussed gaps and potential for future research.

Materials and methods
To test our hypothesis that the depth distribution of SOC follows the same pattern as that of roots, we reviewed literature on the profile distribution of fine roots (FRs) and SOC. Preference was given to published literature with means along with their standard errors and sample sizes, which allowed us to recalculate the values for FRB and SOC by depth (in g/m^2). Where it was necessary to combine data, for instance for fine roots which had been split into two size classes (0 – 1 and 1 – 2 mm) for each given depth of sampling into one size class, new standard errors for the combined means were calculated following the approach outlined by Utts and Heckard (2007). For studies where matching the depth segments was not possible, symbols were appended to the mean values and their standard errors (both for FRB and SOC). The source(s) of data used to recalculate the values for these parameters are denoted by symbols in the first columns of tables 1 and 2.

Results and discussion
Profile distribution of fine root biomass for selected landuse systems
The profile distribution of FRB for defined landuses or plant species presented (Table 1), indicated that depth distribution of FRB varied with landuse but generally, over 60% of all live root biomass occurred above 0.1 m depth. Interestingly, the pattern of distribution of dead root biomass with depth (data not presented) was more related to that of FRB. Kirsi and Sisko (1999) explained that although fine roots account for only 15 – 20% of root biomass in forests, their growth and maintenance can account for as much as 76% of the total

net primary production. Rooting depth and profile distribution may be used to indicate potential to locate and sequester SOC in subsurface horizons (Rasse *et al.* 2005; Denef and Six 2006*)*, because during their growth, roots punch through moist soil deforming and repacking it (Hinsinger *et al.* 2009), while depositing C along their way (Hirth *et al.* 2005).

Table 1. Profile distribution of fine root biomass of selected landuse systems.

	Fine root (≤ 2 mm) biomass g/m^2 by soil depth (m)					
Landuse	0.0 – 0.2	0.2 – 0.4	0.4 – 0.6	0.6 – 0.8	0.8 – 1.0	> 1.0
YFS (14 – 16 yr)[†]	308±48.76	161±13.34	283±138.62	25±8.6		
TFS (26 – 29 yr)[†]	286 ±110.45	226±92.48	139±98.49	103±30.89		
OFS (114 – 121 yr)[†]	262±62.13	317±25.02	218±46.32	24±5.83		
Scots pine[††]	61.7±4.13	32.8±3.02	12.5±2.00			
Festuca grassland[†††]	925±199**	645±94**	301±77**	114±30**	91± 59**	67.5± >18**
Stipa grassland[†††]	297±58**	174±18**	73±10**	79±15**	98± 48**	51±>8**
Desert[†††]	216±49**	84±29**	17±5**	3±1**	0±0**	5±>2**
[a]Temp grasslands[‡]	980	238	126	42	14	
Tropic grassland[‡]	798	336	126	84	42	
Crops[‡]	93	18	15	6	4.5	
Native pasture[‡‡]	90±5.67^	22±6^	10±3.2^	10±2.8^	8±4.6^	36±>5.1^
Sown pasture[‡‡]	134±6.0^	10±1.4^	8±3.4^	6±2.2^	6±3.4^	10.6±8.2^
Native Vertosol[‡‡]	97±11.55^	38±8.2^	18±8.0^	8±3.8^		
Native Chromosol[‡‡]	107±28.21^	10±4.2^	10±3.8^	4±2.2^	6±2.78^	

Data recalculated from [†]Park *et al*. (2007), depths: 0 – 0.1, 0.1 – 0.3, 0.3 – C horizon and C horizon, respectively. YFS, TFS and OFS stand for young, transitional and old forest stands, respectively; [††]Vanguelova *et al*. (2005), depths: 0.0 – 0.15, 0.15 – 0.45 and 0.45 – 0.6, respectively; [†††]Schulz *et al*. (1996), data from an aridity gradient in Patagonia, **depths: 0.0 – 0.1, 0.11 – 0.3, 0.31 – 0.5, 0.51 – 0.75, 0.76 – 1.0 and > 1.0 m; [‡]Jackson *et al*. (1996) (FRB) and Jobbagy and Jackson (2000) (percentage distribution of the biomass by depth); [‡‡]Lodge and Murphy (2006), ^depths: 0.0 – 0.05, 0.05 – 0.1, 0.1 – 0.3, 0.3 – 0.5, 0.5 – 0.7, 0.7 – 0.9, 0.9 – 1.1, >1.1 m. Since the biomass of 0.0 – 0.05 and 0.05 – 0.1-m depth was pooled together, it also necessitated computing the respective combined standard errors following the same procedure in Utts and Heckard (2007).

Depth distribution of soil organic carbon

The distribution of SOC by profile depth for defined landuses or biomes (Table 2) shows that SOC tends to be concentrated in the topsoil where the highest FRB is located. We found a broad similarity between SOC distribution by depth and that of FRB. This relationship is supported by a number of conceptual models which seem to indicate that roots are not only the principal source of SOC but that root-OC is more stable in the soil than shoot-OC. Tisdall and Oades (1982) proposed a hierarchical model of soil aggregate development in which roots are the dominant structures holding together aggregates at the highest and most complex level of organisation. Gale *et al*. (2000) demonstrated how small soil macroaggregates and large microaggregates, held together by root-particulate organic matter (RPOM), could withstand slaking more than aggregates devoid of RPOM, a key factor in the stabilisation of SOC. Watteau *et al*. (2006) advanced the models of Tisdall and Oades (1982) and Gale *et al*. (2000) and observed that decay and water uptake could occur simultaneously in the "coarse" roots of maize (*Zea mays* L.). They also observed how silt- and clay-sized aggregates were drawn, along with water, toward central cylinders of decomposing coarse roots for distances of up to 15 µm, a process they postulated could initiate the formation of soil aggregates. Moreover, many fine roots were colonised by bacteria, whose decomposition upon death resulted in granulofibrillar residues which formed associations with silt and clay minerals. Therefore, roots are not just simple structures holding preformed aggregates together but roots act as centres for the formation of aggregates and nucleation of SOC in such aggregates (Watteau *et al.* 2006). Methodological advances that are being made in root studies with regard to their exploration and modification of the soil matrix should shed more light on their roles that have continued to elude rhizosphere scientists.

There is a dearth of published literature in which the depth distribution of SOC was simultaneously compared with profile distribution of FRB and FR morphological properties. Moreover, the existing literatures reported results in various non-standradised units, making it difficult to make comparisons across landuses and biomes. In some cases results from one study were presented in different sections in various journals, instead of in a consolidated article in a single journal publication. In some literature, crucial information was not declared, including sample sizes used, soil bulk density data where OC was given as %, which made it difficult to express SOC per-area-basis. Still in other literature, data on FRB and SOC was

concealed in graphs, percentages, or never presented at all. The depths of measurement of the parameters also varied tremendously, as shown in the Tables 1 and 2.

Table 2. Profile distribution of soil organic carbon (SOC) in selected landuse systems or biomes.

	SOC g/m^2 by depth (m)					
Landuse	0.0 – 0.1	0.1 – 0.2	0.2 – 0.3	0.3 – 0.5	0.5 – 0.8	0.8 – 1.0
Eucalyptus forest[††]	5100	3600	2700	4500	4200	2800
Grassland[††]	4700	3000	2300	3600	1600	900
Native pasture[‡]	900		220*	100*	360*	360*
Sown pasture[‡]	1340		100*	80*	240*	30*
[a]Native Chromosol[‡]	1070		100*	100*	160*	20
Native pasture[G]	240		1990**	14.6**		13.1**
Pine plantation[G]	17.4		16.4**	1330**		1020**
Rice-berseem[‡‡]		3050[đ]	2350[đ]	1700[đ]		
[b]KBS – LTER, MI[†††]		2140[đđ]		520[đđ]	260[đđ]	
[c]Hoytville, OH[†††]		3560[đđ]		1720[đđ]	860[đđ]	
[d]Placerville, CA[†††]		3620[đđ]	3060[đđ]		1460[đđ]	
Temperate forests[†]		4830^	2150^	1270^	830^	630^
Tropical forests[†]		8370^	4790^	3590^	2720^	2280^
[e]Temp grasslands[†]		4310^	2420^	1580^	1260^	950^
[f]Tropic grasslands[†]		7130^	4550^	3560^	2570^	1980^
Boreal forest[†]		5600^	2800^	1460^	780^	560^
Deserts[†]		3700^	2460^	2020^	1680^	1460^
Crops[†]		6440^	3610^	2360^	1880^	1410^
Tundra[†]		4560^	3310^	2170^	800^	570^

The data used to compute SOC stocks were derived from: [†]Jobaggy and Jackson (2000) and Jackson *et al*. (1996), [††]Chen *et al*. (2005), [†††]Paul *et al*. (2006), [‡]Lodge and Murphy (2006), [‡‡]Majumda *et al*. (2008), [G]Guo *et al*. (2008). The depths of SOC values with symbols were different from those in the row just below the title "SOC Mg/ha by depth (m)" such that: *0.1 – 0.3, 0.3 – 0.5, 0.5 – 0.9 and 0.9 – > 1.1; **0.1 – 0.3, 0.3 – 0.6, 0.6 – 1.0; [đ]0.0 – 0.2, 0.2 – 0.4 and 0.4 – 0.6; [đđ]0.0 – 0.2, 0.25 – 0.5 and 0.5 – 1.0; ^0.0 – 0.2, 0.2 – 0.4, 0.4 – 0.6, 0.6 – 0.8 and 0.8 – 1.0 m. The SOC values for the terrestrial biomes denoted by ^are in Pg (1 Pg = 10^{15} g) recalculated from the data in[†], and all the rest in Mg/ha Abbreviations: [a]Native pasture on Chromosol, [b]Kellog Biological Research Station – Long-term Ecological Research, MI (Michigan State, USA), [c]Hoytville, OH (Ohio State, USA), [d]Placerville, CA (Canada, USA), [e]Temperate grasslands, [f]Tropical grassland.

Conclusion and research imperatives

There appears to be a relationship between the profile distribution of fine roots and depth distribution of SOC. However, simultaneous site- and landuse-specific studies comparing the depth distribution of roots and that of SOC are needed to generate empirical data to ascertain this relationship. Existing theories underpin roots for their dual role in the formation and stabilisation of soil aggregates on the one hand and the nucleation of SOC in such aggregates potentially for long-term storage belowground on the other hand. A standard procedure for data collection, interval depths of sampling, units of measurement and presentation format is needed to enable comparison of relationship between FRB and SOC under different systems. It is proposed that the type and minimum amount of data which should accompany future communications on depth distribution of FRs and SOC submitted for publication should be established to allow for consistency in presentation and interpretation of results without violating statistical principles and intellectual property issues.

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Root development of young citrus trees in soil fertilized with phosphorus

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Abstract
Increased plant growth and fruit production of citrus trees occur in soils with high fertility. Phosphorus (P) is a key element for nutrient management, for which adequate supply and soil distribution is required in planting groves. The response of young plants of Pêra sweet orange on two contrasting rootstocks to P fertilization was studied in an experiment conducted in mini-rhizotrons under a screen house. Results demonstrated greater growth of plants on Rangpur lime compared to those on Cleopatra mandarin which corresponded to greater root development as evaluated by root growth rate and architecture. These parameters varied according to P availability in soil.

Key Words
Citrus, rootstock, growth rate, soil fertility, nutrient availability.

Introduction
The present study is part of a major ongoing project to evaluate phosphorus (P) absorption, use efficiency and distribution of P-forms in citrus trees. Earlier research work has demonstrated that limited P availability of low fertility tropical soils, predominant in Brazil, impairs citrus production (Quaggio *et al.* 1998, 2002). Furthermore, growth and fruit yield of citrus trees in response to P fertilization greatly vary according to scion/rootstock combinations (Quaggio *et al.* 2004), what is marked for sweet oranges on Cleopatra mandarin. Trees on Cleopatra appear to be less efficient for P absorption and therefore require additional nutrient supply compared to those either on Rangpur lime or Swingle citrumelo rootstocks (Mattos *et al.* 2006, 2009). This distinct requirement results from physiological and morphological plant characteristics that affect P acquisition from soil and use by plant (Ragothama 1999). However, specific mechanisms that explain such responses for cultivated citrus are not fully understood yet. Additionally, adequate P rates and fertilizer placement in the soil profile are particularly important before tree planting in the field, mostly because of specific adsorption of P-$H_2PO_4^-$ by Fe and Al-oxides what reduces nutrient availability to plants. Based on the above, the objectives of this study were to evaluate the response of Pêra sweet orange grafted on two contrasting rootstocks to P distribution in two layers of the soil profile of mini-rhizotrons by measuring root growth rate, dry mass production and P absorption by plants.

Methods
Young plants of Pêra sweet orange [*Citrus sinensis* (L.) Osb.] grafted on Rangpur lime (*C. limonia* Osb.) or Cleopatra mandarin (*C. reshni* hort. ex Tanaka) were grown in mini-rhizotrons containing 25 kg of soil and maintained under screen house. The rhizotrons in this study were constructed with 1.0 m height and 0.25 m diameter polyvinyl chloride (PVC) half tubes to form a semicircle along its axis. A transparent glass plate (4.0 mm thick) was installed to the flat side of the rhizotron to allow roots to be observed. The mini-rhizotrons were positioned at 80º from the ground level to force roots to grow toward the transparent plate. This plate was covered with a flexible black Plexiglas one, which was only removed for the observation of root development during the course of the study.The experiment was arranged on a 2 x 5 factorial design with treatments defined by the combination of rootstocks and soil P distribution replicated three times. A sandy clay loam Oxisol (333 g/kg clay and 617 g/kg sand) with pH (0.1 mol/L $CaCl_2$) = 5.6 and CEC = 76.5 $mmol_c/dm^3$ collected from the surface layer of a degraded pasture land was used. Portions of the soil were moistened and incubated in plastic bags for 30-d after application of P, K, and micronutrients. Phosphorus was applied as $NH_4H_2PO_4$ (MAP) at three different rates and N supplied was balanced with application of $CaCl_2$ as required. After incubation, soil P extracted by anionic resin levels (Raij *et al.* 1986), in mg/dm^3, were: P_0 = 4 (no P applied), $P_{0.5}$ = 12, P_1 = 19 and P_2 = 47. Two 45 cm soil layers were disposed in the mini-rhizotrons in order to simulate P distribution in the soil profile as follow: P_0/P_0, P_1/P_0, $P_{0.5}/P_{0.5}$, P_2/P_0, and

P_1/P_1 before plant transplant. The mini-rhizotrons were irrigated with application of water to soil surface and subsurface; in this later case, a perforated PVC tube was installed at 45 cm soil depth was used. Water amounts were applied to replenish evapotranspiration losses.

After 15 days of plant transplant in the mini-rhizotrons, up to 15 root tips visualized on the transparent plate were marked for measurement of root length after 7-day intervals until 84 days. Total measured root length was determined for each treatment and daily root growth rate was calculated. Number of root tips visualized from the 21st to the 84th day after plant transplant was counted and number of fully expanded leaves was evaluated at the 84th day. Plants were destructively harvest 134 days after transplant in the mini-rhizotrons and separated into leaf and stem. Roots were separated from each soil layer soil using a sieve. Plant material was washed in di-water and dried at 60±2 ºC for 48 h for dry weight determinations. A simple analysis of variance (ANOVA) was used to test the hypothesis that means for root growth rate, number of root tips and leaves per plant were equal (*Prob.* = 0.05) using the GLM procedure of the SAS® system (SAS Institute 1996). The study is still in progress for evaluation of P content and uptake efficiency by plants after destructive harvest.

Results

Average root growth rate of Pêra sweet orange on Rangpur lime rootstock was 0.34 cm/day, whereas the same for plants on Cleopatra mandarin rootstock was only 0.24 cm/day (Table 1). Similarly, plants on Rangpur lime showed greater number of root tips and leaves per plant (68 and 22, respectively). These differential responses correlate with observed tree development in the field (Pompeu Jr. 2005).

The significant interaction (*Prob.* < 0.01) between rootstocks and soil P suggested that plants on Cleopatra mandarin lime are responsive to increased P availability in the soil according to increased root growth rate and number of root tips observed in the mini-rhizotrons, even though the same appeared to be less efficient for P acquisition compared to those on Rangpur lime rootstock (Table 1). These results are in line with study conducted in the field for nonbearing Valencia and Natal sweet oranges trees (Mattos Jr. *et al.* 2006).

Plant development was greater with the P_2/P_0 treatment as measured by leaf number per plant (Table 1), what suggested that increased P-fertilizer application to surface soil may overcome difficulties of P incorporation at deeper soil layers in the field similarly to the P_1/P_1 treatment. However, this later need to be validated during long term field experiments.

Table 1. Growth characteristics of young plants of Pêra sweet orange on different rootstocks as affected by soil resin-P availability in mini-rhizotrons.

Rootstock combination	Soil P treatment([1])	Root growth rate([2])	Root tips([3])	Leaves([4])
		cm/day	# per plant	# per plant
Pêra/	P_0/P_0	0.42	94	20
Rangpur lime	P_1/P_0	0.31	46	20
	$P_{0.5}/P_{0.5}$	0.27	52	22
	P_2/P_0	0.42	94	26
	P_1/P_1	0.26	55	21
Mean values		0.34	68	22
Pêra/	P_0/P_0	0.14	27	12
Cleopatra mandarin	P_1/P_0	0.22	29	12
	$P_{0.5}/P_{0.5}$	0.21	28	13
	P_2/P_0	0.30	38	14
	P_1/P_1	0.24	33	14
Mean values		0.22	31	13
Prob. < F				
Rootstock (RS)		17.76**	19.90**	51.02**
Soil P (SP)		0.28^{ns}	0.03^{ns}	2.21^{ns}
RS*SP		6.87**	7.88**	0.01^{ns}

([1]) resin-P in each of 45 cm soil layer, in mg/dm^3, P_0 = 4, $P_{0.5}$ = 12, P_1 = 19 and P_2 = 47.
([2]) mean values for data evaluated at 7-day intervals from 14 to 84 days after transplant in mini-rhizotrons.
([3]) mean values for data evaluated at 7-day intervals from 21 to 84 days after transplant in mini-rhizotrons.
([4]) number of leaves per plant after 84 days of transplant.

The response of plants on Rangpur lime at the P_0/P_0 treatment suggested that roots of this rootstock variety present greater plasticity at limited P availability in the soil by changing its architecture (Robinson 1994;

Lynch and Brown 1997). Physiological characteristics may also be involved, however further studies are necessary to better clarify this question. Root growth was greater for plants on the Rangpur lime rootstock (Table 2), which was proportional to the leaf and stem dry matter production. Maximum growth was observed with the P_2/P_0 treatment, whereas the minimum was with the P_0/P_0. Plants on the Cleopatra mandarin differed from those on the Rangpur lime since growth corresponded to soil-P levels with maximum observed with the P_1/P_1 treatment, in which greater amount of P was better distributed in the rhizotrons profile. Furthermore, plants on the Rangpur lime presented lower root to shoot ratio in the P_0/P_0 treatment (0.77) compared to those on Cleopatra (0.96) what together with data presented for root growth rate and number of root tips observed in the vertical plane of the rhizotrons (Table 1) suggested that Rangpur lime presents more plastic roots for P uptake at limited availability in the soil (Ragothama 1999). Treatment effects on P accumulation will be further discussed.

Table 2. Dry matter production of young plants of Pêra sweet orange on different rootstocks as affected by soil resin-P availability in mini-rhizotrons.

Rootstock combination	Soil P treatment([1])	Dry matter([2])					Root:shoot ratio
		RootU	RootL	Leaf	Stem	Total	
		(---------- g ----------)					
Pêra/	P_0/P_0	6.03	2.59	6.09	5.20	19.91	0.77
Rangpur lime	P_1/P_0	7.58	1.88	5.32	5.58	20.35	0.90
	$P_{0.5}/P_{0.5}$	7.23	2.04	4.03	4.88	18.18	1.04
	P_2/P_0	12.18	4.91	11.17	8.83	37.07	0.89
	P_1/P_1	8.08	2.58	9.25	6.57	26.48	0.67
Mean values		8.22	2.80	7.17	6.21	24.40	0.85
Pêra/	P_0/P_0	3.72	0.75	2.45	2.26	9.18	0.96
Cleopatra mandarin	P_1/P_0	4.16	1.07	3.42	2.60	11.24	0.88
	$P_{0.5}/P_{0.5}$	4.85	0.76	3.52	2.99	12.12	0.87
	P_2/P_0	5.32	1.56	4.61	3.39	14.88	0.85
	P_1/P_1	6.86	1.35	4.30	3.02	15.53	1.15
Mean values		4.98	1.10	3.66	2.85	12.59	0.94
Prob. < F							
Rootstock (RS)		77.06**	21.73**	92.51**	84.64**	138.64**	1.93^{ns}
Soil P (SP)		13.52**	3.37**	19.60**	5.63**	19.15**	0.89^{ns}
RS*SP		7.05**	1.47**	8.61**	2.56*	7.46**	3.03*

([1]) resin-P in each of 45 cm soil layer. in mg/dm^3. $P_0 = 4$. $P_{0.5} = 12$. $P_1 = 19$ and $P_2 = 47$.
([2]) RootU and RootL = refer to roots in the upper and lower 45 cm soil layers. respectively. harvested 134 days after transplant in mini-rhizotrons.

Conclusion

Young plants of Pêra sweet oranges on Rangpur lime demonstrated more vigorous growth compared to those on Cleopatra mandarin. This was correspondent to observed root system characteristics, which growth rate, architecture and plant dry matter production varied depending on P availability in the soil.

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Root growth of upland rice genotypes as influenced by nitrogen fertilization

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Abstract
The Plant root system is an important organ which supplies water and nutrients to plants. Information is limited on influence of nitrogen fertilization on upland rice root growth. A greenhouse experiment was conducted to evaluate the influence of nitrogen fertilization on root growth of 20 upland rice genotypes. The N rates used were 0 mg/kg (low) and 300 mg/kg (high) of soil. Nitrogen X genotype interactions for root length and root dry weight were highly significant ($P < 0.01$), indicating that differences among genotypes were not consistent at two N rates. Overall, greater root length and root dry weight were obtained at an N fertilization rate of 300 mg/kg compared with the 0 mg N/kg soil. However, genotypes differ significantly in producing root length and root dry weight. Nitrogen fertilization produced fine roots and more root hairs compared with control treatment. Based on root dry weight efficiency index (RDWEI) for N use efficiency, 70% genotypes were classified as efficient, 15% were classified as moderately efficient and 15% were classified as inefficient. Root dry weight efficiency index trait can be incorporated in upland rice for improving water and nutrient efficiency in favor of higher yields.

Key Words
Oryza sativa L., Oxisol, root dry weight efficiency index, shoot-root ratio.

Introduction
Root system is an important organ of plants. It absorbs water and nutrients from soil and supply to plant tops for their metabolic activities. Roots also give plants a mechanical support and supply hormones which help plants in many physiological and biochemical processes associated with growth and development. A vigorous root system is responsible for development of healthy plants and consequently higher yields. Roots which are left in the soil after crop harvest, improve soil organic matter content, nitrogen cycle and microbial activities (Sainju *et al.* 2005). All these activities improve soil structure, soil water holding capacity, water infiltration rate in the soil and reduce soil bulk density and soil erosion which ultimately leads to higher soil productivity. Root growth is controlled genetically and also influenced by environmental factors. Environmental factors which influence plant root growth are soil temperature, soil moisture content, solar radiation and soil physical, chemical and biological properties (Fageria 2009). Most of the root biomass of annual crops is located in the 0-20 cm soil depth. This may be associated with large amount of organic matter, nutrient accumulation and water availability in the top soil layer compared to lower soil depths (Sainju *et al.* 2005). In most of the research articles data related to plant tops growth and development are presented. Research data related to growth and development of crop root systems are limited. The objective of this study was to evaluate influence of nitrogen fertilization on root growth of twenty upland rice genotypes.

Materials and methods
The experiment was conducted in the greenhouse of National Rice and Bean Research Center of EMBRAPA, Santo Antôio de Goias, Brazil. The soil used in the experiment was an Oxisol. The genotypes used were: BRA01506, BRA01596, BRA01600, BRA02535, BRA02601, BRA032033, BRA032039, BRA032048, BRA032051, BRA042094, BRA042156, BRA042160, BRA052015, BRA052023, BRA052033, BRA052034, BRA052045, BRA052053, BRS Primavera, BRS Sertaneja. The N levels used were low (0 mg/kg) and high (300 mg/kg) and supplied through urea. Half of the N was applied at sowing and remaining half as topdressing 45 days after sowing. Four plants were maintained in each pot after germination. Experiments were conducted in plastic pots with 9 kg of soil in each pot. At the time of sowing, each pot received 200 mg P as triple super phosphate, 200 mg K as potassium chloride and 10 mg Zn/kg of soil as zinc sulfate. Each pot also received 10 g dolomitic lime four weeks before sowing. The liming material was having 33% CaO, 14% MgO and 85% neutralizing power. The pots were subjected to wetting and drying cycles. Experimental design was a complete block with three replications. Pots were water everyday to maintain soil moisture at about field capacity during growth cycle. Shoot and grain were separated at harvest and material was dried in an oven at 70 ^{0}C to a constant weight. Top-root ratio was

calculated by using the following formula (Fageria 1992):

$$\text{Top-root ratio} = \frac{Tops\ dry\ weight\ (grain\ plus\ straw)}{Roots\ dry\ weight}$$

Root dry weight efficiency index (RDWEI) for N use efficiency of genotypes was calculated using the following formula as proposed by Fageria (2009):

$$\text{RDWEI} = \frac{Root\ dry\ weight\ at\ low\ N\ rate}{Average\ root\ dry\ weight\ of\ 20\ genotypes\ at\ low\ N\ rate} \text{ X } \frac{Root\ dry\ weight\ at\ high\ N\ rate}{Average\ root\ dry\ weight\ of\ 20\ genotypes\ at\ high\ N\ rate}$$

Genotypes having RDWEI values >1 were classified as efficient, genotypes having RDWEI values, between 0.5 and 1 were classified as moderately efficient and those with RDWEI values < 0.5 were classified as inefficient in N use. At the time of harvesting, roots from each pot were removed manually to determine maximum length and dry weight. Roots were washed in water including distilled water several times before drying to a constant weight. Data were analyzed by analysis of variance and means were compared by Tukeys test at the 5% probability level.

Results and discussion

Root length

Nitrogen X genotype interaction was significant for root length and root dry weight; therefore data are reported separately for two N rates (Table 1). Root length varied from 27 cm produced by genotype BRA01600 to 43 cm produced by genotype BRA032039, with an average value of 30.49 cm at the low N rate (0 mg N/kg). Similarly at high N rate (300 mg N/kg), root length ranged from 21 cm produced by genotype BRA01506 cm to 40.33 cm produced by genotype BRS Sertaneja, with an average value of 31.99 cm. Overall, root length was 5% higher at the higher N rate compared to lower N rate. However, 35% genotypes produced lower root length at the higher N rate compared to lower N rate. Fageria (1992) reported higher root length of rice at low N rate compared to high N rate in nutrient solution. Fageria (1992) also reported that at nutrient deficient levels, root length is higher compared to high nutrient levels because tendency of plants to tap nutrients from deeper soil layers.

Root dry weight

Root dry weight varied from 0.87 g/plant produced by genotype BRA01596 to 1.78 g/plant produced by genotype BRA052034, with an average value of 1.38 g/plant at the lower N rate (0 mg N/kg). At the higher N rate (300 mg N/kg), root dry weight ranged from 0.40 g/plant produced by genotype BRA01506 to 4.14 g/plant produced by genotype BRA052023 g/plant, with an average value of 2.72 g/plant. Overall, root dry weight was 97% higher at the higher N rate compared to lower N rate. Fageria and Baligar (2005), and Fageria (2009) reported that N fertilization improved root dry weight in crop plants, including upland rice. The positive effect of N o root dry matter has been previously documented (Fageria 2009).

Visual evaluation of root growth

Overall, root growth was reduced in the 0 mg N/kg treatment compared to 300 mg N/kg of soil. However, there were differences among genotypes in root growth visually observed as length as well as weight. For example, root growth of genotypes BRA052015 was having minimum difference in length as well as weight at two N rates. Under low N rate, roots were thicker and having less hairs compared to higher N rate. Roots were thinner and root hairs were also fine at the high N rate compared to lower N rate. The root diameter and hairs may be indicative of the nutrient absorption capacity of the root system (Fageria 2009). Thinner roots with fine fine hairs can absorb more nutrients and water compared to thicker roots with less fine hairs. These results concur with published results on the effects of N fertilization on root characteristics (Drew *et al.* 1993).

Classification of genotypes for N use efficiency

Based on RDWEI, most efficient genotypes in N use efficiency were BRA052045, BRA052023, BRA052033, BRA042160, BRA032039, BRA052015, BRA052034, BRA052053, BRA02601, BRA02535, BRS Primavera, BRA042094, BRS Sertaneja and BRA032048. The three genotypes moderately efficient in N use efficiency were BRA032051, BRA042156 and BRA032033. Remaining three genotypes which were

Table 1. Root length and root dry weight of 20 upland rice genotypes as influenced by nitrogen fertilization.

Genotype	Root length (cm)		Root dry weight (g/plant)	
	0 mg N/kg	300 mg N/kg	0 mg N/kg	300 mg N/kg
BRA01506	34.67ab	21.00cd	0.92a	0.40f
BRA01596	30.00b	15.67d	0.87a	0.45f
BRA01600	27.00ab	25.33bcd	1.14a	1.03ef
BRA02535	31.67ab	30.00abcd	1.33a	3.25abcd
BRA02601	28.00b	32.67abc	1.11a	3.73ab
BRA032033	30.00a	28.00abcd	1.12a	2.41cd
BRA032039	43.00a	34.67abc	1.24a	3.77ab
BRA032048	28.50b	32.00abc	1.05a	3.62abc
BRA032051	30.67ab	35.67ab	1.31a	2.82bcd
BRA042094	30.33b	38.00ab	1.78a	2.31de
BRA042156	29.33b	33.00abc	1.19a	2.84bcd
BRA042160	29.67b	32.50abc	1.51a	3.33abcd
BRA052015	31.00ab	35.00abc	1.67a	2.83bcd
BRA052023	29.67b	26.50abcd	1.48a	4.14a
BRA052033	30.33b	31.33abc	1.56a	3.38abcd
BRA052034	29.00b	37.67ab	1.78a	2.58bcd
BRA052045	28.67b	37.67ab	1.75a	3.66abc
BRA052053	29.00b	36.50ab	1.65a	2.72bcd
BRS Primavera	29.33b	36.33ab	1.68a	2.49bcd
BRS Sertaneja	30.00b	40.33a	1.45a	2.68bcd
Average	30.49	31.99	1.38	2.72
F-Test				
N rate (N)	NS		*	
Genotype (G)	**		**	
N X G	**		**	
CV(%)	14.25		15.86	

**Significant at the 5 and 1% probability levels, respectively. Means followed by the same letter in the same column are not significant at the 5% probability level by Tukey test.

inefficient were BRA01506, BRA01596 and BRA01600. Limited data are available in the literature for classification of upland rice genotypes on the basis of root growth and hence, can not be compared with the published work.

Conclusions

Knowledge of root growth is important to know the ability of a crop to tap soil nutrients and water necessary to sustain plant growth. A significant interaction between genotypes and N rates was found for root growth because some genotypes were highly response to the N application while others were not. Thus genotypes selection for N use efficiency is an important aspect for improving root growth and consequently yield of upland rice in Brazilian Oxisols. Nitrogen fertilization produced thinner roots with fine root hairs compared with without N fertilization treatment. The genotypes were classified in three groups based on root dry weight efficiency index. Among 20 genotypes evaluated, 70% fell into efficient group, 15% fell into moderately efficient group and 15% fell into inefficient group.

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Selection of dark septate endophytes from *Ericaceae* plants to enhance blueberry (*Vaccinium corymbosum* L.) seedling growth

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Abstract
An experiment was conducted to find a beneficial root endophyte in order to enhance blueberry biomass accumulation as well as to confirm the role of dark septate endophyte (DSE) under axenic condition. The plant material for DSE isolation was selected from the *Ericaceae*. A total of 91 isolates were obtained from 300 root sections of *Rhododendron pulchrum*, *Rhododendron obtusum* and *Pieris japonica*. There were no significant differences observed after 10 weeks of inoculation on shoot length, root length and fresh weight of the seedlings, however there was a tendency of having longer root in the seedlings with some isolates especially with Pj029 (78.6 mm) and Pj022 (68 mm) compared with the control (51 mm). Nine putatively benefical endophytes were subjected to phylogenetic analysis. Based on the homology search the Rp005 isolate from *Rhododendron pulchrum* host was identified as *Heteroconium chaetospira*. Three other isolates namely, Rp022 and Rp011 isolates both from *Rhododendron pulchrum* host and Pj023 isolate from *Pieris japonica* host were confirmed as *Leptodontidium orchidicola*. These isolates have the ability to form inter- and intracellular hyphal structure within the host epidermal cell and assume to have bidirectional nutrient flow between the host and endophyte.

Key Words
Dark Septate Endophyte, *Ericaceae*, blueberry, colonization, intracellular structure

Introduction
The dark septate endophytes (DSE) are broadly classified as conidial, sterile septate fungal endophytes that form melanised structures such as inter- and intracellular hyphae and microsclerotia in the plant roots and known to have affinities with ascomycetes (Jumpponen and Trappe 1998). Despite the wide host preference of DSE, there are many basic questions that should be addressed (Jumpponen 2001). The ecology of DSE is largely unknown and hypotheses are based on sparse evidence. The range and relative importance in *Ericaceae* are still unclear (Hambelton and Currah 1997) and thus, research should focus on the functional aspects of the interaction between the two organisms involved in the association. In this study, a survey was conducted among some of the native *Ericaceae* species of the Japanese flora to isolate root associated fungal endophytes that probably can enhance the growth of blueberry seedling as an initial step toward to the better upland soil adaptability of blueberry. In addition, we examined the ability of these isolates to form intracellular structures with the host and we characterized the community structure of those endophytes.

Methods
Root sampling
Root samples were collected from the following ericaceous species: *Rhododendron obtusum* (Lindl.) Planch. *Rhododendron pulchrum* Sweet and *Pieris japonica* (Thunb.) D. Don ex G. Don. in the Azalea-garden of Graduate School of Horticulture, Chiba University, Japan (35°46'N, 139°54'E). The sterilized root segments were placed in 90 x 15 mm Petri dishes filled with malt extract agar (MEA, 2%) and incubated at 18°C for 1 month. Only the dark, slow growing and septate endophytes were selected and subcultured to MEA media.

Inoculation test
The blueberry seeds were extracted from a commercial available frozen blueberry fruits. After the surface sterilization, the seeds were placed on PDA for germination and individually transplanted to 20 ml test tubes filled with 10 ml of modified Mitchell & Read media. Then, seedlings were inoculated with each of the 91 isolates and one additional known DSE *Heteroconium chaetospira* provided by Narisawa *et al.* (2000). Control tubes (non-inoculated) were also prepared containing only the host. Ten weeks after inoculation, the seedlings were destructively harvested to measure fresh weight and length of shoot and root. The roots were stained with trypan blue and determined root colonization by endophytes. Data on colonization intensity was measured using the modified method of Giovannetti and Mosse (1980).

DNA extraction, sequencing and phylogenetic analysis
The genomic DNA of isolates was extracted by FastDNA®Kit following the manufacture's instructions. The ITS region was amplified by ITS1F and ITS4 primers. The amplification was carried out in an automated PCR Thermal Cycler TP-600. The PCR product was purified with SUPREC™-PCR purification column. The sequencing reaction mixture was prepared with BigDye terminator cycle Sequencing Kit followed by the recommendation of manufacturer. ITS3 primer was used to span the 5.8S and ITS2 region. The sequencing data of isolates were subjected for homology search using the BLAST program through the GenBank database to match the closest sequence. Phylogenetic relation between the isolates of the present study and the closest sequences from related studies were combined to create a neighbor-joining tree by ClustalW. The neighbor joining tree was compiled by TREEVIEW. The confidence levels were calculated from 1000 replicates bootstrap samplings.

Results
Inoculation test
There were no significant differences observed after 10 weeks of inoculation on shoot length, root length and fresh weight of the seedlings, however there was a tendency of having longer root in the seedlings with some isolates especially with Pj029 (78.6 mm) and Pj022 (68 mm) compared with the control (51 mm). The highest colonization intensity was observed in Rp005 a *C. chaetospira* isolate (10.1%) followed by the Rp022 isolate (8.1%) and confirm this finding with the formation of ERM like intracellular structure within the epidermal cells in both strains.

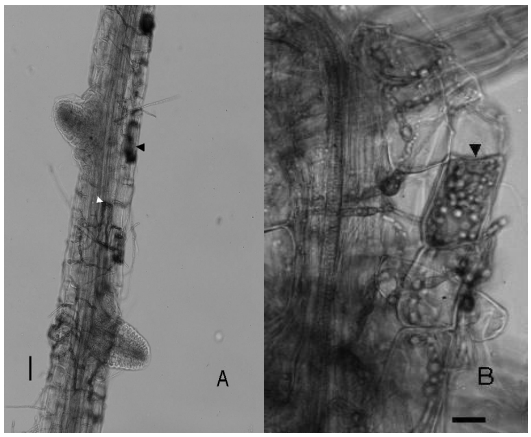

Figure 1. (A) Chain-like hypha of Rp005 form intercellular (white arrowhead) and intracellular (black arrowhead) structures in the epidermal cells of blueberry (*Vaccinium corymbosum* L) seedling. Bar is 100µm. (B) Epidermal cells of blueberry (*Vaccinium corymbosum* L.) seedling heavily colonized (arrowhead) by hyphal complexes of Rp011. Bar is 30µm.

Phylogenetic analysis
The highly diverse ITS2 region of the rDNA was used to determine relationships of isolates at species level. The neighbor joining tree clustered the isolates into three distinct clades. Because the internal branches having weak bootstrap support between clade I and the other two clades (clade II and clade III), indicating unrelated polyphyletic origin of isolates among the clade I and in the rest two clades. The Rp005 isolate have a high sequence similarity with *Cladophialophora chaetospira* (EU035406) and clustered in a strong bootstrap supported monophyletic clade (clade II) with other *C. chaetospira* strains including the *Heteroconium chaetospira*. The phylogenetic positions of Rp011, Rp022 and Pj023 were set to individual clade (clade III) having strong bootstrap support with a DSE of *Ranunculus adoneus* (*Ranunculaceae*), with an *Epacris* (*Ericaceae*) root associated fungi and with other *Leptodontidium orchidicola* strains. A strong bootstrap supported cluster was clamped the Ro012 with Ro024. Both show significant bootstrap support with *Cyphellophora* sp. Isolates Pj022 and Pj029 did not have any significant homology during BLAST search and their identity still unknown and will remain so until isolates will sporulate or until more taxa will be sequenced. Based on sequence similarity and phylogenetic analysis, five isolates were confirmed as DSE, including the *Heteroconium chaetospira* (syn. *Cladophialophora c*.) isolate derived from Narisawa *et al* (2000). The taxonomy of Rp005 with 100% sequence similarity confirmed as *C. chaetospira* isolate was from the host plant *R. pulchrum*. Moreover, distinctive colonization intensity was observed between these *H. chaetospira* (by Narisawa) and *H. chaetospira* Rp005 isolates 2.5% and 10.1 %, respectively. Usuki and

Narisawa (2004) found no statistical differences in the colonization intensity of axenic *R. obtusum var. kaempferi* (*Ericaceae*) seedlings inoculated with four strains of *H. chaetospira* (DSE) that varied between 14.5 to 19.5 %. Further, the same authors addressed that one strain of *Oidiodendron maius* (ERM) produced significantly higher percentage of intercellular structure (31.8%) compared to the above mentioned four *H. chaetospira*. Similarly to our study, seedling fresh weight of *Rhododendron obtusum var. kaempferi* did not show any significant difference. Dalpé (1986) found a similar percentage of colonization by five *Oidiodendron* strains that ranged between 3-21% in axenic blueberry (*Vaccinium angustifolium* Ait.) experiment after 60 days of inoculation. Narisawa (2007) observed that the *H. chaetospira* is a commonly occurring DSE, in contrast with what was previously reported. Other more frequently isolated DSE species such as *Phialocephala fortinii* and *L. orchidicola* have faster growth rate, and thus, make it more difficult to detect and isolate *H. chaetospira*. In this study, both *H. chaetospira* isolates have the ability to form intracellular structure in blueberry host that resembled ERM coil. The formation of intracellular structure in *Ericaceae* by *H. chaetospira* was previously reported in axenic *Rhododendron. obtusum var. kaempferi* (Usuki and Narisawa (2004), however, the source of Rp005 isolate derived from *R. pulchrum* in our study, indicates the first isolation of *H. chaetospira* from an *Ericaceae* in Japan. Interestingly, there was no any report about DSE species in a complete endophytic survey of *R. obtusum var. kaempferi* at approximately 30 km NE from the sample site of the current study (Usuki *et al.* 2003).

Table 1. Results of inoculation test. Effects of different *Ericaceae* derived endophytes on the root lenght, shoot lenght, fresh weight, formation of intracellular structure and colonization intensity of blueberry (*Vaccinium corymbosum* L.) seedlings 10 weeks after inoculation.

Isolates†	Shoot lenght (mm)	Root lenght (mm)	Fresh weight (mg)	Formation of intracellular structure	Colonization intensity (%)
Rp005	28.3 a	51.6 a	65.0 a	+	10.1 a
Rp011	41.0 a	43.3 a	48.5 a	+	1.8 cd
Rp022	29.6 a	48.3 a	61.6 a	+	8.1 a
Ro012	46.6 a	50.3 a	70.1 a	+	5.2 bcd
Ro024	38.3 a	49.6 a	56.4 a	-	0.0 d
Ro034	36.0 a	58.3 a	69.5 a	-	0.0 d
Pj022	46.0 a	68.0 a	93.4 a	-	0.0 d
Pj023	39.6 a	63.3 a	65.4 a	+	1.2 d
Pj029	48.3 a	78.6 a	66.1 a	+	4.1 abc
H.chaetospira	47.6 a	57.6 a	63.6 a	+	2.5 bcd
Control	35.6 a	51.0 a	76.0 a	-	-

* same letter means not significantly different at $P \leq 0.05$ based on Tukey Test
†Rp=*Rhododendron pulchrum*, Ro=*Rhododendron obtusum* Pj= *Pieris japonica*.

The Rp022, Pj023 and Rp011 isolates set in a monophyletic position in clade III which dominated by *L. orchidicola*. The high percentage of sequence similarity with other strains of *L. orchidicola* (98%) obtained from BLAST search among GenBank data and strong bootstrap support of the clades suggested the conspecificity of these three isolates with *L. orchidicola*. The *L. orchidicola* recovered from both *Rhododendron pulchrum* (Rp011 and Rp022 isolates) and *Pieris japonica* (Pj023 isolate) in our study confirmed the previous findings of Midgley *et al.* (2004) where they find two different species of neighboring Ericaceae host plants (*Woollsia pungens* and *Leucopogon parviflorus*) sharing same endophyte. Inspite of the worldwide distribution of *Ericaceae*, only 25 species has been mentioned as host of DSE (Hambelton and Currah 1997, Currach and Tsuneda 1993, Ahlich and Sieber 1996). Majority of the hosts were sampled in alpine heath, a stable sand dune and an ombrotrophic *Sphagnum* bog in the Canadian Rocky Mountains (Hambelton and Currah 1997), a *R. obtusum* plant in Tottori, Japan and in a coniferous forest in Switzerland, Central-Europe (Ahlich and Sieber 1996). All sampled sites were covered by ericaceous shrub understory. Solely the *Phialocephala fortinii* represented the DSE in all sites irrespective of host plants or geographic location. The Rp005 isolate *(H. chaetospira)* from *R. pulchrum* host the first report of a *Heteroconium* strain derived from an *Ericaceae* in Japan. The Rp022 and Rp011 isolates from *R. pulchrum* and Pj023 isolate from *P. japonica* host the first *L. orchidicola* strains from *Ericaceae*.

Conclusion

Our result suggests that both *H. chaetospira* (Rp005) and *L. orchidicola* (Rp022, Pj023 and Rp011) have the

ability to form inter- and intracellular structures in axenic blueberry seedlings together with their enzymatic abilities (Fernando and Currach 1995) and in broader perspective, possibly act on the part of ericoid mycorrhiza and make them a good candidate for further container experiments with blueberry plants in order to clarify the function in host-endophyte continuum.

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Shrub-interspace dynamics of soil microbial communities under different patch areas in a semiarid river valley, SW China

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Abstract
In arid and semi-arid ecosystems, belowground characteristics are influenced by the formation and persistence of "fertile islands" in contrast to barren plant interspaces. We investigated the soil properties of patched vegetation, especially the belowground properties of microbial communities, by directly comparison of soil microbial communities between soils under patched vegetation and in the interplant spaces with increased patch areas in Minjiang River arid valley. We found "fertile islands" exist in patch soils, and the increased substrates utilization ability of soil microbes may indicate a positive feedback to maintain the "fertile islands" under patched vegetation. Under the patches, soil C, soil N, soil microbial biomass, soil water content, clay concentration were significantly improved by the increasing vegetation patch areas. The fungus to bacteria ratio was increasing with the enlarged vegetation and the contribution of fungi to microbe was closely relevant to patch area. Compared the barren plant interspaces, soil nutrient condition and microbial biomass were significantly influenced by its adjacent patch. Thus, "the islands of fertility" may be beneficial and advance the rehabilitation of plant in this semi-arid region.

Key Words
Arid vegetation, fertile islands, soil microbes, patch.

Introduction
Soil degradation is a critical environmental problem in arid and semiarid ecosystems. The establishment of plant cover is reported to be one of the most effective means for erosion control and regeneration of the degraded soil (Xu *et al.* 2008; Garcia *et al.* 2002). Plant cover can reduce water runoff and sediment loss, and favour soil-development processes by improving soil organic matter, soil structure, soil water and nutrient-holding capacity, which are especially important in improving semiarid soil quality. Soil quality and its degradation depend on a large number of physical, chemical, biological, microbiological and biochemical properties, and the last two are the most sensitive since they respond rapidly to changes. It is widely accepted that high levels of microbial activity are fundamental in maintaining soil quality. In arid and semi-arid ecosystems, belowground characteristics are influenced by the formation and persistence of "fertile islands" in contrast to barren plant interspaces. Soil microbes may respond differently to "fertile" and interspace soils. In the few studies assessing microbial community composition between shrub and interspace soils, the importance of shrub-islands in structuring communities was evident (Ewing *et al.* 2007; Aandernd *et al.* 2008). Relative to interspace soils, shrub soils have higher total soil C and N, support more microbial biomass, and are associated with higher C and N mineralization rates (Schaeffer and Evans 2005; Ewing *et al.* 2007). Microbial community composition may not be regulated primarily by soil C, but by N and P availability and soil stresses (i.e. high B and Cl concentrations) in shrub soils, or by soil stresses in interspace soils (Aandernd *et al.* 2008). The establishment of suitable plant cover could improve the physico-chemical and biological properties of soil. Soil microbes are supposed to respond quickly to these changes. However, how soil microorganisms in the "fertile islands" as well as the barren soils interact with aboveground plant cover remains unclear. Because vegetation in arid and semiarid ecosystems is typically patchy and most of these systems experienced degradation, researches on shrub-interspace soil microbial responses to plant cover in the restoration processes is meaningful. In the upper minjiang river, vegetation (mostly shrub) is typically patchy, and we take patch area (%) to indicate plant cover. We hypothesized that "fertile islands" existed under patched vegetation, and microbial communities would respond differently in shrub and interspace soils under different plant covers.

Methods
Site description and sampling
The study area (31°47′54″-31°47′54″N, 103°44′30″-31°44′40″E) is part of the dry-warm valley of the upper

Minjiang River, one of the four principal tributaries of the Yangtze River. Vegetation here was damaged years ago and now is undergoing revegetation. The mean annual precipitation is 494 mm and the mean annual evaporation is 1,332 mm. The aridity index for this area is within 1.5–3.49, which is typical of semiarid environments (Xu *et al.* 2008). The predominant soil type is calcic cambisols. Regional vegetation mainly consists of small-leaf arid shrubsand sparse grasses (Ma *et al.* 2004; Xu *et al.* 2008). We selected four typical locations with an increased patch areas (30%, 40%, 45%, 75% of the study area respectively) in the core area of the valley. Topography conditions are almost the same. The dominant species are *Sophora. viciifolia* and *Artemisia gmelinii* for 30% patched*; A. gmelinii* for 40% patched; *A. gmelinii, Onosma mertensioides* and *Wikstroemia stenophylla* for 45% patched; *Bauhinia faberi* and *A. gmelinii* for 75% patched. Three 5m*5m plots were randomly selected for each site. In September 2009, soil samples were collected from surface soils (0-10cm) under different dominant species and interspace soils in each site. Each soil sample consisted of five sub-samples removed with a soil corer (3.5cm in diameter) and thoroughly mixed to obtain a composite sample. Prior to transport, all soil samples were stored at approximately 4℃ immediately after sampling.

Soil physico-chemical measurements
Soils were sieved to 2mm and litter and roots retained on sieve were separated by hand. The methods used to measure soil physico-chemial properties were as described by Xu *et al.* 2008.

Soil microbial measurements
Microbial biomass C and N were determined on 10g soil using the fumigation-extraction procedure described by Macdonald et al 2009. Substrate introduced respiration (SIR) was assessed using the MicroResp^{TM} CO_2 detection system (Campbell *et al.* 2003). To cope with experimental artifacts when dealing with calcareous soils derived from $CaCO_3$-CO_2-H_2O equilibrium and acidic substrates, we followed the recommendations from Oren and Steinberger (2008). Microbial community structure was assessed by PLFA analysis using a modified method (White *et al.* 1979) based on that of Bligh and Dyer (1959). Briefly, soil samples from each site were freeze-dried; finely ground and 4g were used for extracting PLFAs. The separated fatty acid methyl-esters were identified and quantified by GC-MS (HP6890/MSD5973) using methyl nonadecanoate (19:0) as the internal standard. The specific fatty acids used to indicate bacteria, fungi, Gram-positive bacteria, Gram-negative bacteria, Actinobacteria were as described by Zachary *et al.* (2008) and Macdonald *et al.* (2009).

Statistical analyses
Data was analysed using both univariate and multivariate methods. Effects of patch area on soil properties and microbial communities were determined using one-way ANOVA and Tukey's HSD test at $P < 0.05$. When necessary, data were log or arcsine transformed to accomplish the assumptions of normality and homogeneity of variances of ANOVA. All univariate analyses were conducted using SPSS13.0 (SPSS Inc., IL. Chicago, USA). And linear model redundancy analysis (RDA) was used to test the relationships between ecosystem characteristics and the distribution of microbial groups. The significance of the canonical axis was tested using the Monte Carlo permutation test. And all multivariate techniques were performed with CANOCO software (Canoco for Windows 4.5).

Results
Microbial communities between patch and interspace soils
Compared with interspace, patch soils were higher in GWC, Clay%, nutrients, microbial biomass, lower in pH(Table 1, Figure 1), and "fertile islands" existed in patch soils. SIR showed that microbial activities were stronger in patch compared to interspace, which may indicate faster nutrient cycling in patch soil (data not show). RDA analysis showed that patch area and C/N explained large variance in microbial groups for both shrub and interplant soils; GWC explained little variance in shrub soils while in interspace soils, Actinobacteria, Gram-positive bacteria, total bacteria and total PLFA were negatively related to GWC (Figure 3). Soil microbes in the interspace soils were not regulated by GWC but nutrients to a large extent.

Microbial communities within patch soils
Large patch area (i.e. 45% or 75%) increased GWC, Clay%, nutrients and microbial biomass and lowered pH (Table 1, Figure 1). The significant effect of patch area was also found in the increased utilization of carbon sources like carbohydrate, carboxylic acid and amino acid ($P<0.05$) (data not show). Mol% of fungi was higher in 30% patched than 40%, but lower than 75% patched. Actinobacterial increased with increasing

patch area; the same tendency was also mirrored in gram-negative bacteria gram-positive bacteria (Figure 2 D). The fungus to bacteria ratio was generally increased with the enlarged vegetation ($P<0.05$) (data not shown) and the contribution of fungi to microbe was closely relevant to patch areas (Figure 3 B). GWC and TP were two main constraints in the study area (Song *et al.* 2009). However, the variance of GWC and TP was slight and explained less variance in RDA analysis (Table 1, Figure 3 B).

Table 1. Soil physico-chemical properties. Values are means. Different letters in the same column indicate significant differences ($P<0.05$) under different patch areas. Abbreviations are as follows: GWC= soil water content, SOC= soil organic carbon, C/N =SOC/TN, TN= total nitrogen, TP= total phosphorus.

	Area (%)	GWC (%)	pH	TC (g/kg)	TN (g/kg)	TP (g/kg)	SOC (g/kg)	C/N	Clay (%)	Silt (%)	Sand (%)
	30	4.41a	8.65a	8.92b	1.17b	0.40a	8.01c	6.62b	1.29b	82.03a	16.68a
Inter-	40	3.49a	8.65a	20.32a	2.18a	0.48a	17.18c	7.74ab	1.54b	77.56a	20.91a
space	45	2.49b	7.17b	20.35a	2.17a	0.44a	19.78ab	8.99a	1.49b	78.18a	20.33a
	75	2.72c	8.17a	28.32a	2.51a	0.47a	25.91a	8.77a	1.62a	80.55a	17.84a
	30	4.62b	8.23a	15.63c	1.73c	0.47a	13.86b	8.01c	1.41b	83.50a	15.54a
Patch	40	4.20b	8.67a	25.30bc	2.49bc	0.47a	22.05b	8.83b	1.34b	77.01a	21.65a
	45	5.07a	7.19b	32.64ab	3.06b	0.53a	31.58a	10.24a	1.49b	78.48a	20.03a
	75	6.09a	7.54b	42.02a	3.93a	0.52a	39.39a	9.77a	1.71a	78.91a	19.38a

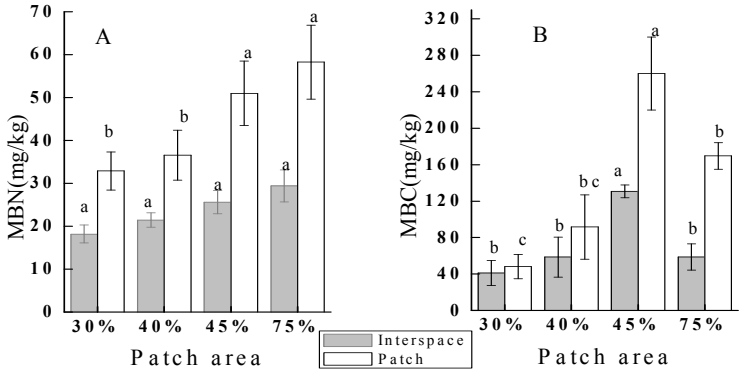

Figure 1. Microbial biomass C (A) and N (B) in patch and interspace soils respectively. Values are means with standard error. Different letters indicate significant differences ($P<0.05$).

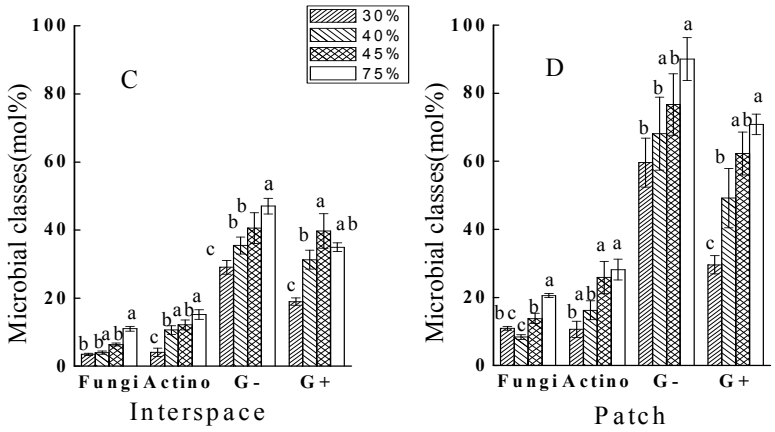

Figure 2. Distribution of microbial groups in interspace (C) and patch soils (D) respectively. Mole % of all microbial groups was based on the total nmol PLFA g^{-1} soil. Values are means with standard error. Different letters indicate significant differences ($P<0.05$).

Microbial communities within interspace soils

Soils in large patch area (i.e. 45% or 75%) increased in nutrient content, Clay%, microbial biomass and decreased in pH (Table 1, Figure 1). The overall carbon utilization ability also increased (data not show). However, GWC decreased slightly. Soil microbes in the interspace soils were not regulated by GWC but nutrients to a large extent (Figure 3, A). Microbial groups responded differently to patch area (Figure 2). However, Changes in fungi behaved in a similar way as patch soils (Figures 2 and 3).

Conclusion

"Fertile islands" existed in the patches and microbial activity may promote the maintenance of these "islands". Within the patches, soil quality was significantly improved with increased vegetation patch areas; the fungus to bacteria ratio was increasing with the enlarged vegetation patch. For plant interspaces, soil nutrient condition and microbial biomass were significantly influenced by its adjacent patch. Thus, "the islands of fertility" may be beneficial and advance for the rehabilitation of plant in this semi-arid region.

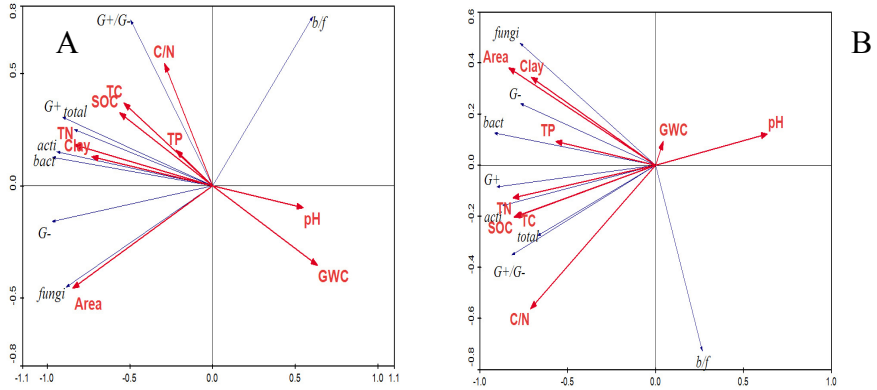

Figure 3. RDA analysis between soil properties (plus patch area) and microbial group characteristics in interspace (A) and patch soils (B) respectively. Red arrows represent soil properties (plus patch area), and blue represent microbial group characteristics. For abbreviations in red arrows see Table 1; abbreviations for blue arrows are as follows: total=total PLFA; bact=bacterial PLFA; acti=actinobacterial PLFA; b/f= bacterial PLFA/fungi PLFA.

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Soil fungal communities along land use gradients of three German biodiversity exploratories: A comparison of classical cloning and sequencing approach with high throughput sequencing

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Abstract
Our research is carried out in the frame of the Biodiversity Exploratory initiative, an interdisciplinary consortium granted by the German Science Foundation (DFG). Our knowledge and view of soil microbial diversity is changed in the last decades due to the advance in the sequencing technology. We test several molecular techniques ranging from classical cloning and sequencing over screening of metagenomic fosmid libraries to high throughput 454 sequencing to investigate soil fungal diversity in forest and grassland ecosystems of the German Biodiversity Exploratories. The results and implications of these comparisons will be presented and discussed.

Key Words
Fungal diversity, metagenomic library, laccase gene

Introduction
The Biodiversity Exploratories is a research project comprising three research sites, called Exploratories, across Germany. The project provides the opportunity to investigate the relationship between biodiversity of different organism groups, the role of land use for biodiversity and the role of biodiversity in ecosystem processes. Each Exploratory comprises grassland and forest ecosystems each with 1000 plots, which are further rescaled to 100 experimental plots and 18 very intensive plots reflecting land use gradients and different land use intensity levels. The Biodiversity Exploratories is a platform for an interdisciplinary research including abiotic and biotic soil ecology projects. Within the biotic soil ecology project consortium our focus is to investigate soil fungi and fungal enzymes using PCR-based, metagenomic and high throughput sequencing techniques.

Among soil microorganisms, fungi play a major role in degradative processes and their ability to produce oxidative enzymes such as laccases (EC 1.10.3.2) and laccase-like multicopper oxidases (LMCO) contributes to high ecological importance especially for lignin and polyphenol degradation (Baldrian 2006; Chen *et al.* 2001; Leonowicz *et al.* 2001). Polymerase chain reaction (PCR) based approaches have recently been used to investigate soil fungal diversity and community composition of different ecosystems, which avoids limitations associated with traditional culture-based techniques (e.g. Kirk *et al.* 2004). Actually two approaches coexist, the use of structural markers on the one hand and functional markers on the other hand. The internal transcribed spacer (ITS) region surrounding the 5.8S ribosomal DNA became the most commonly used structural marker for fungal species identification and diversity studies (Martin and Rygiewicz 2005). In contrast to studies based on structural markers for the detection of the whole fungal community, the use of functional genes encoding enzymes that catalyze key steps in biochemical pathways allows detection of specific functional groups involved in a particular biogeochemical process (Chen *et al.* 2001; Zak *et al.* 2006). Ecological studies using fungal laccase or LMCO genes showed their wide occurrence and role in the primary attack of recalcitrant soil organic matter by breaking down crosslinks and aromatic ring structures (Baldrian 2006; Kirk and Farrell 1987). Due to the fact that fungal laccases are widespread exoenzymes with low substrate specificity and broad ecological impact, they have been frequently used as functional marker genes in monitoring diversity of fungi with oxidative potential in different soils (Lauber 2009; Luis *et al.* 2004). Despite several advantages of such approaches they also reflect certain limitations and our knowledge about key ecological questions could also be expanded using new molecular techniques like metagenomics and metatranscriptomics, which allows investigating different genes and their products within multispecies communities.

Our study presents one of the first comparison of soil fungal communities revealed by PCR based methods using primer pairs targeting the ITS rDNA fragment as structural marker and fungal laccase genes as functional marker. Additionally, we constructed soil metagenomic fosmid libraries and we will screen them for fungal laccase genes and will also employ a 454 deep sequencing of the fungal ITS rDNA. These datasets will be analysed with soil and vegetation metadata to assess the ecological implication of the fungal species and laccase gene diversity in the functioning of these ecosystems.

Methods

Study site and sample collection

Soil cores were taken in April 2008 from very intensive plots (VIP) of forests and grasslands of the three biodiversity exploratories (http://www.biodiversity-exploratories.de). From each of the plots soil cores (8.3 cm in diameter), excluding the organic soil layer, were taken down to the parent rock material. The respective mineral soil horizons of the cores were combined into a composite sample and stones were separated from the bulk soil. The management and soil types of the plots are given in Figure 1.

Plot	Management	Soil type
SEG1	fertilized meadow (low moor)	Histosol
SEG2		Histosol
SEG3		Histosol
SEG4	mowed pasture (low moor) without fertilization	Histosol
SEG5		Histosol
SEG6		Histosol
SEG7	pasture (low moor)	Histosol
SEG8		Histosol
SEG9		Histosol

Plot	Management	Soil type
HEG1	meadow mowed 3 times per year and fertilized	Cambisol
HEG2		Stagnosol
HEG3		Stagnosol
HEG4	mowed and fertilized pasture with cattle	Stagnosol
HEG5		Stagnosol
HEG6		Stagnosol
HEG7	pasture with cattle and without fertilization	Stagnosol
HEG8		Stagnosol
HEG9		Stagnosol

Plot	Management	Soil type
AEG1	meadow mowed 2-3 times per year and fertilized	Leptosol
AEG2		Leptosol
AEG3		Leptosol
AEG4	mowed and fertilized pasture with cattle and horses	Leptosol
AEG5		Leptosol
AEG6		Leptosol
AEG7	pasture with sheep and without fertilization	Leptosol
AEG8		Leptosol
AEG9		Leptosol

Plot	Management	Soil type
SEW1	Pine monoculture	Arenosol
SEW2		Arenosol
SEW3		Arenosol
SEW4	Pine-Beech forest	Arenosol
SEW5	Beech forest	Arenosol
SEW6		Arenosol
SEW7	Natural beech forest	Arenosol
SEW8		Albeluvisol
SEW9		Arenosol

Plot	Management	Soil type
HEW1	Spruce monoculture	Cambisol
HEW2		Cambisol
HEW3		Luvisol
HEW4	Beech age class forest	Leptosol
HEW5		Cambisol
HEW6		Luvisol
HEW7	Beech selection cutting forest	Luvisol
HEW8		Luvisol
HEW9		Luvisol
HEW10	Natural beech forest	Luvisol
HEW11		Luvisol
HEW12		Luvisol

Plot	Management	Soil type
AEW1	Spruce age class forest	Cambisol
AEW2		Cambisol
AEW3		Cambisol
AEW4	Beech age class forest	Cambisol
AEW5		Cambisol
AEW6		Cambisol
AEW7	Natural beech forest	Cambisol
AEW8		Cambisol
AEW9		Cambisol

Figure 1. The three Biodiversity Exploratories are located at: Schorfheide-Chorin 52° 57′ 0″ N, 13° 37′ 0″ E, Hainich 51° 1′ 17″ N, 10° 30′ 36″ E, Schwäbische Alb 48° 34′ 58.8″ N, 9° 30′ 5.4″ E. Tables show very intensive plots (VIP) and their corresponding management and soil types. Sites are designated by location (S-Schorfheide-Chorin, H-Hainich, A-Schwäbische Alb, E-Experimental plot, W- Forest and G-Grassland).

Classical PCR-cloning-sequencing

Genomic DNA was isolated from 0.5 g of soil and stone samples using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories Inc. Carlsbad, CA, USA) as recommended by the manufacturer. The ITS region of the ribosomal DNA and the laccase gene were amplified with the primer pairs ITS1F and ITS4 (Martin and Rygiewicz 2005) and Cu1F and Cu2R (Luis *et al.* 2004). The purified products of ITS and laccase genes were ligated into a pCR4-Topo vector and transformed using TOP10 chemical competent *E. coli* cells according to the instructions provided with the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany). Colonies were checked for inserts with M13F and M13R primers. Sequencing was done on an ABI PRISM 3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with M13F as sequencing primer. Both ITS and laccase gene sequences were grouped into operational taxonomic units (OTU) using online tool FastGroup II (http://biome.sdsu.edu/fastgroup/fg_tools.htm) at 97% and 99% similarity, respectively, according to Yu *et al.* (2006). The resulting taxonomic units were checked for their identity against the National Centre for Biotechnology Information (NCBI, GenBank) database using the BLAST search algorithm for identification (Altschul *et al.* 1997).

Metagenomic library construction and screening
Metagenomic libraries were constructed from soil genomic DNA extracts, sheared and gel extracted for fragment length of 35 – 40 Kbp genomic DNA. The DNA fragments were packed in pCC1FOS™ Fosmid vector and titrated to library pools following the CopyControl Fosmid Library Production protocol (http://www.epibio.com). Fosmid library pools are screened by PCR for fungal laccase genes using the primer pair Cu1F/Cu2R. For the isolation of positive clones from complex DNA libraries the method of Hrvatin and Piel (2007) is used.

High throughput amplicon sequencing
The ITS2 fragment of the ITS rDNA will be amplified using tagged fungal specific primers. PCR products will be cleaned and sequenced using the 454 titanium amplicon sequencing kit and the 454 Genome Sequencer (Roche Applied Science).

Results and discussions
Our preliminary data showed that the fungal diversity revealed with structural (primer pair ITS1F/ITS4) and functional marker genes (primer pair Cu1F/Cu2R) differs and considering the effect hierarchy (e.g. forest type, soil type, soil chemical parameters) the two approaches showed different patterns. Additionally we will focus on the interpretation of fungal communities in different habitats (bulk soil and stones). We will present the complete comparative analysis of the three approaches in revealing soil fungal communities. Correlation of the fungal diversity data with the different management levels and environmental variables will be presented and discussed in detail.

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Soil without plants: the consequences for microorganisms and mesofauna

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Abstract
Plants provide the primary carbon source for soil communities, but there are few studies on the consequences of their absence over an extended period. It is unclear whether a reduction in the number of organisms associated with diminishing resources is associated with a reduction in their diversity. To investigate this, soil organic carbon (SOC), microbial and mesofaunal communities in the Highfield Ley-Arable Experiment at Rothamsted Research were compared. Samples were taken from an old grass sward, a section converted to arable rotation 60 years ago, and a section regularly tilled to maintain a bare fallow for the past 50 years. Microbial biomass is positively correlated with SOC regardless of the specific composition of the carbon, but the diversity of the community does not appear to be influenced by these parameters. In contrast, the abundance and diversity of the soil mesofauna are strongly influenced by recent plant inputs. On the fallow plot, the virtual elimination of fresh carbon inputs results in the degradation of SOC and reduction in microbial and mesofaunal numbers. However, the plot supports a species-rich and metabolically active bacterial community that is not significantly reduced in diversity compared to soil under arable cultivation or maintained as grass.

Key Words
Soil management, ley-arable rotation, community structure, diversity index

Introduction
Carbon inputs to soil from recent photosynthesis are of similar importance to decomposition of more recalcitrant organic matter in driving biological processes in soil: the plant community influences the community composition of the rhizosphere and soil biota, which in turn influence plant productivity (Wardle *et al.* 2004). Other than a few groups of chemoautrotrophic bacteria, the majority of soil organisms rely on fixed carbon from root exudation and other plant residues together with the excreta and remains of soil fauna and inputs from above-ground mega fauna. To establish the extent to which plant inputs affect below-ground communities, we investigated the long-term Highfield Ley-Arable Experiment at Rothamsted, where parts of a long-standing grass ley were ploughed in 1949 and managed subsequently by arable rotation. In addition, since 1959, one area has been maintained, by regular tillage, as bare-fallow. The composition of organic inputs is determined by the plants that are present, so a mixed grass sward is likely to provide a more complex substrate than a wheat monoculture. The amount of soil organic carbon (SOC) in soil is a balance between what plants supply to the soil through the roots and, in managed systems, crop removals, any returns of straw or stubble, and the decomposition of SOC already present (the 'native' pool). Previous work at this site comparing the arable soil with permanent grass has graphically illustrated the decline of SOC over time associated with conversion from grass to arable cultivation (Johnson *et al.* 2009). We tested the hypothesis that the bare-fallow soil supports smaller and less diverse microbial and mesofaunal communities than soil from the more substrate rich arable and grass plots.

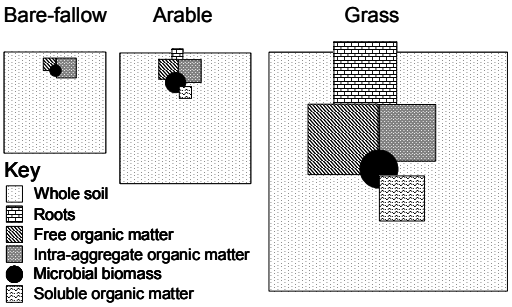
Methods
Site and sampling regime
Highfield soil is a silty loam over clay (Batcome series) - a Chromic Luvisol by FAO criteria. Prior to the summer harvest, we sampled: (i) soil continuously under arable crops in rotation (winter wheat at time of sampling) since 1949; (ii) soil from an adjacent area of unmanaged mixed grass sward unchanged for at least 200 years: (iii) soil from an adjacent bare-fallow strip which has been maintained crop- and weed-free since 1959 by tillage whenever weed growth was apparent (minimum three times a year). Arable crops were managed as normal farming practice, receiving NPK fertilizer, fungicides and insecticides as required. Lime had previously been applied to maintain the pH at c 6.0, although pH had been allowed to decline in the decade prior to this sampling; fertilizer had last been applied to the arable crop two months prior to sampling. An earlier investigation (Watts and Dexter 1997) found that the soil under permanent grass contains much more organic carbon than that under arable and bare-fallow, and that soil physical properties follow the same

trend: aggregate stability and water retention were found to be greatest under grass and least under bare-fallow. The sampling strategy, SOC)and its sub-fractions, soil pH, the total microbial biomass and its diversity, the abundance of heterotrophic bacteria and the sub-group pseudomonads, soil mesofauna (collembola and mites belonging to the Oribatei or other groups), phospholipid fatty acids (PLFAs), Biolog™ substrate utilisation by active bacteria, 16S rRNA and DNA abundance and diversity using DGGE were measured as described by Hirsch *et al.* (2009). The MultiVariate Statistical Package MVSP version 3.13d (Kovach Computing Services) was used to estimate Shannon Diversity Indices *H´* from qualitative and quantitative data, PLFA HPLC traces, band position and intensity on DGGE gels, and for Principle Component Analysis (PCA) of this data.

Soil organic carbon fractionation
Soil fractions were separated by their density to reflect the *in-situ* position of the materials within the soil matrix (Sohi *et al.* 2001). The fractions include free organic carbon – discrete organic particles of non-specific size, located outside stable aggregates, and intra-aggregate organic matter – discrete organic particles released from their enclosure within stable aggregates by ultrasonic disruption. The former fraction is assumed to be more available and the latter more recalcitrant to mineralization by the soil microbiota. The sum of these fractions was subtracted from the total SOC to estimate the soluble fraction that includes root exudates and microbial metabolites.

Results

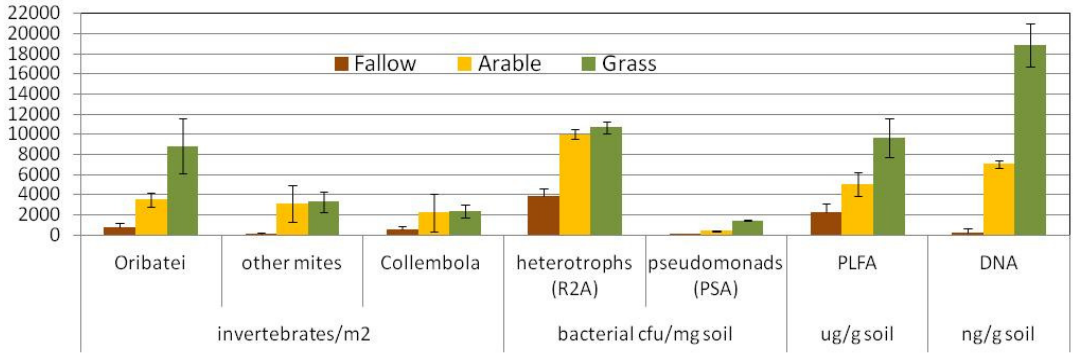
Total soil organic carbon
Arable and old permanent grass soils had similar pH (5.5); the bare-fallow soil a slightly lower (pH 5.1). A higher carbon (C) content of the grass soil (5.39 %) compared to the arable (1.62 % C) and bare-fallow soil (0.97 % C) was consistent with the previous finding that it had a lower aggregate density (Watts and Dexter 1997). The C: N ratio increased bare-fallow<arable<grass, probably reflecting the higher proportion of fresh roots in the grass soil, with no roots detected in the bare-fallow. Soil under grass contained more than three times the SOC concentration than the arable soil in the 0-10 cm layer, i.e. significantly more potential substrate for soil biota; the arable soil contained two-thirds more SOC than the bare-fallow soil. In the arable soil, C inputs are mixed through the top 23 cm by annual tillage; in the bare-fallow soil the minimal input from occasional weeds is similarly homogenised by more frequent tillage. Thus, after almost 50 years without significant plant input, the bare-fallow soil contains ~ 30 t C ha^{-1} as recalcitrant SOC, <8% of that in the original grassland soil. Figure 1 illustrates graphically the relative differences in SOC and the proportions of different fractions, most obviously the relative decline in free SOC and corresponding increase in the more recalcitrant intra-aggregate C fraction when grass, arable and bare-fallow soils are compared in that order, but microbial biomass C remained a fairly constant proportion of the total SOC. The biomass-specific respiration was higher, and similar, in grass compared to bare-fallow and arable soils.



The area of the large squares represents the relative amount of total organic C in each soil; superimposed on these, the areas of the smaller shapes represent the relative amount of each discrete organic C fraction, assuming that the soil aggregate density is similar to the bulk density and that the C content of root dry matter is 40 % (Robinson, 2001).

Figure 1. Distribution of organic carbon amongst different components of the three soils.

Soil biomass and the relative abundance of biota
Soil biomass analyses (C, respiration, PLFA and DNA yields), numbers of culturable bacteria, and soil invertebrates all reflected the SOC, which provides substrate for the soil biota. The total number of mites showed a similar trend to SOC and biomass, with most in the grass and fewest in the bare-fallow soil (Figure 2). This was clearly demonstrated in the subgroup Oribitei. The other mites and the collembola showed a similar trend but mite numbers were not significantly different between arable and grass soils, and collembola numbers were significantly different only between the grass and bare-fallow soils.

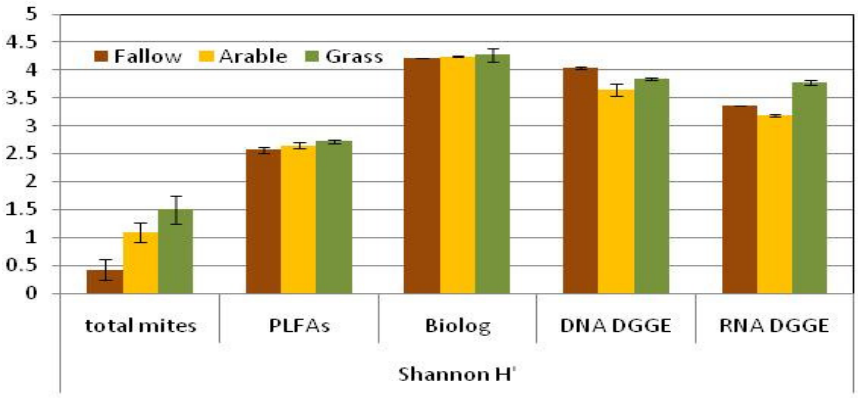


Note differences in scale of each measurement to allow results to be displayed on the same numerical axis. Standard errors of the means are shown.

Figure 2. Relative abundance of mesofauna, culturable bacteria and microbial biomarkers in soils

Estimates of diversity

The Shannon diversity index *H′* of mites was significantly different in the three soils, following the order grass>arable>bare-fallow. However, this marked trend which followed the differences in SOC and biomass was not seen in the other communities (Figure 3). PLFA analysis, which indicates overall soil microbial diversity (bacteria and fungi) followed the same trend, albeit with very small differences, but the Biolog *H′* was not significantly different, possibly because this method is indicative only of the small sub-population that can grow in the different Biolog substrates (Degens and Harris 1997; Nannipieri *et al.* 2003). A further departure from the grass>arable>bare-fallow trend was observed with the DNA and RNA-based methods. More individual 16S DGGE bands arising from DNA, indicating the relative abundance and number of bacterial species, were detected in the bare-fallow soil (61) than in the grass (50) or arable (44) soils. The analysis of rRNA molecules (RNA derived from ribosomes in recently active cells, indicating metabolically active species) reversed this trend, with 31 bands seen in bare-fallow compared with 50 in grass, although arable again had fewest (26). The relative intensity of each band, in addition to the abundance of bands, was used to estimate *H′*, which reflects species abundance (Figure 3).



PCAs based on the relative positions and intensities of bands on DGGE gels differentiated the three soils. The metabolically active species represented by bands from RNA molecules appeared similar to the total species composition represented by DNA for grass, but were distinct and different for the other two soils indicating that each soil has a different active subset of bacteria. Standard errors of means are shown.

Figure 3. Shannon diversity indices, *H'*

Discussion

Total soil organic carbon

The greater abundance of soil mesofauna under grass compared to bare-fallow and arable soils is associated with the availability of plant material, exemplified by the abundance of roots under grass and their absence in the bare-fallow soil. Similarly the larger microbial community in the grass soil may be because substrates are more readily available to microorganisms as well as more plentiful than under arable or bare-fallow soil. By virtue of being in an accessible location within the mineral soil matrix (outside stable soil aggregates), free organic C may reflect the most readily available portion of native SOC. Its presence in the bare-fallow soil is somewhat surprising, given the long-term absence of crops and the rigorous control of weeds and it may comprise relict material rendered inaccessible by the absence of free nutrients. A previous thermo-chemical analysis of the fractions from the same plots indicated that free organic matter in the bare-fallow resembled, chemically, intra-aggregate SOC from typical soils (Lopez-Capel *et al.* 2005) and the dramatic reduction in SOC included changes in the relative amounts of available C in fractions that were free or associated with aggregates, compared the original permanent grassland. The soil that had been reverted to arable cultivation showed a less dramatic but significant reduction in these factors.

Soil biomass and the relative abundance of biota
Biomass measures (C, respiration, total PLFA, total DNA), the number of culturable bacteria, and soil invertebrates all reflected the trend grass>arable>bare-fallow in SOC. The transient weed growth in bare-fallow soil appears to provide only limited resources that support a much-reduced mesofaunal community, including the fungal-feeding collembola and the mites which may be fungivorous, herbivorous or predatory. The pseudomonads are Gram-negative proteobacteria known to flourish in the rhizosphere (Lugtenberg *et al.* 2001), so their paucity in the bare-fallow soil is unsurprising.

Conclusions
Despite differences in community structure there was little to distinguish *H´* in the three soils, whether based on the presence and abundance of individual PLFAs, substrate utilization by active bacteria, or the relative abundance and diversity of total and active bacterial species based on 16S rRNA and DNA. However, the distinct structure of microbial communities in the three soils (in contrast to the similar diversity indicated by *H'*), in particular in the bare-fallow soil that is starved of plant inputs and tilled regularly, demonstrates that the variety of niches available for bacteria. The small inputs of C from soil autotrophs and occasional weeds are sufficient to maintain a functionally diverse community for decades. Results support previous reports that soils under arable cultivation support less diverse microbial communities than those under unimproved pasture (Degens *et al.* 2000; Garbeva *et al.* 2007). Additionally, the results may indicate a significant change in community structure in the absence of a dominant soil biota (in contrast to the grass and arable soils) to one that thrives on the more chemically recalcitrant forms of free C that are present in this starved soil (resembling the intra-aggregate fraction in the other soils), revealing a more specialised albeit genetically diverse community adapted to the recalcitrant intra-aggregate C.

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Spatial distribution of Arbuscular Mycorrhiza (AM) Fungi in the tailing modADA deposition areas

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Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous in soils and establish symbioses with most agricultural plants (Smith and Read 1997). There has been relatively little investigation of the spatial variation in infectivity of AM fungi and its relationship to soil characteristics in mine tailing areas. A study of the spatial variability of AM fungi spores from two natural plant communities showed that the number of spores varied according to plant distribution and soil properties (Carvalho *et al.* 2003). The spatial pattern of mycorrhiza infectivity of AM fungi using a glasshouse bioassay from soils at six sites from a successional chronosequence demonstrated that AM infectivity decreased with time and 44-50% of the total variance in AM infectivity was associated with pseudostripmine site and active soybean field (Boerner *et al.* 1996). In contrast, a bioassay assessment of the infectivity of AM fungi in a forest soil was uniform, which may indicate that the actual scale of the patches of infectivity need to be determined (Jasper *et al.* 1991). Geostatistics provides tools to describe spatial variation and to extend point samples through spatial interpolation (Goovaerts 1999). They have been used to analyze the spatial distribution of soil microorganisms especially in illustrating that soil microorganisms are structured at different spatial scales (Wollum and Cassel 1984; Webster and Boag 1992; Robertson and Freckman 1995; Boerner *et al.* 1996; Robertson *et al.* 1997; Cannavacciuolo *et al.* 1998; Klironomos *et al.* 1999), as are the distributions of microorganisms on the soil surface (Rossi *et al.* 1992).

Materials and Method

In this study, the spatial distribution of the infectivity of AM fungi has been examined in the tailing deposition areas of Freeport Indonesia Mining Company in Timika-Papua. Some soil properties such as soil moisture content, soil pH and soil phosphorus were also examined. The percentage of root colonization by AM Fungi was examined from the root of plants growth in the areas. A geostatistical technique was used to examine the distribution of the infectivity across the tailing areas.

Results

Percentage of Root Colonized by AM Fungi and Spore Number

Table 1 shows the percentage root colonized by AM fungi and the number of spores across the tailing deposition areas. The percentage of root length colonized by AM Fungi range from 0 to 45.08%. Most of the plants growth in the tailings areas were infected by AM Fungi, however no colonization was found in the root of *Phragmites karka* which is one of the plant used commonly in tailing reclamation areas. The highest infection by AMF was in the legume crops (45.08%). The number of AM Fungi spores across the tailing ModADA areas range from 1-17 spores/100 gr soil.

Table 1. The summary of statistics for %RLC and spore numbers of AM fungi in tailing ModADA deposition area

Variable (n=198)	Mean	Median	Standard Deviation	Kurtosis	Skewness	Min	Max	CV (%)
% RLC	21.19	22.77	11.42	-0.54	-0.43	0.00	45.08	53.92
Number of Spores	6.55	6	3.82	-0.17	0,70	1	17	29.84

Soil pH in this areas ranges from 4.65 to 8.67 and the soil phosphorus range from 45 ppm to 101 ppm. The high of soil phosphorus in some sample points across the tailing areas tend to mask the number of spores and % RLC for AM fungi. However, the texture of tailing also affected the distribution of AM fungi in this areas in which the fine texture of the %RLC was higher than for the coarse texture of the tailings.

Spatial Model

The semivariogram model of % RLC and Spore Number of AM fungi is presented in Table 2. The semivariogram model of AM fungi infectivity and spore number was spherical with the distance >450 m and the spatial dependence was considered strong (19%) for % RLC and medium (48%) for number of spores.

Table 2. The semivariance analysis of % RLC and spore numbers of AM fungi in tailing modADA deposition areas

Variable (n=198)	Nugget (Co)	Sill (C=Co+(C1))	Range (m)	Relative Nugget Effect (Co/C)	Spatial Dependence (C1/C)	Model
% RLC	59.78	73.86	1581.13	0.81	0.19	Spherical
Number of Spores	3.86	7.39	486.20	0.52	0.48	Spherical

In general, the percentage of root infection was correlated with the distribution of vegetation and soil texture in the tailings areas. In the fine texture areas the percentage of infection was higher than for coarse soil texture.

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The effect of PGPR strain on wheat yield and quality parameters

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Abstract
N_2-fixing and P-solubilizing bacteria are important in plant nutrition increasing N and P uptake by the plants, and playing a significant role as plant growth-promoting rhizobacteria (PGPR) in the biofertilization of crops. In 2007 and 2008, a field study was conducted in two different locations (Erzurum and Ispir) in the Eastern part of Turkey for investigating the effects of two N_2-fixing and P-solubilizing PGPR strains alone and in combinations on plant yield and nutrient content of wheat in comparison to control and optimum and half doses of N fertilizer application under field condition. All inoculations and fertilizer applications significantly increased grain, total biomass yields, and macro and micro nutrients of wheat over the control. Mixed PGPR inoculations with the strain of OSU-142 + M-13 + *Azospirillum* sp.245 has significantly increased grain yield of wheat as good as full doses of nitrogen. All bacterial inoculations especially mixed inoculation, significantly increased uptake of macro-nutrients (N, P, K, Ca, Mg and S) and micro-nutrients (Fe, Mn, Zn, and Cu) of grain, leaf, and straw part of the plant. The data suggested that seed inoculation with OSU-142 + M-13 + *Azospirillum* sp.245 may substitute N and P fertilizers in wheat production.

Key words
Inoculation; grain yield, plant growth-promoting rhizobacteria; macro and micro element

Introduction

Nitrogen and phosphorus are known to be essential nutrients for plant growth and development. Intensive farming practices that achieve high yield require chemical fertilizers, which are not only costly but may also create environmental problems. The extensive use of chemical fertilizers in agriculture is currently under debate due to environmental concern and fear for consumer health. Consequently, there has recently been a growing level of interest in environmental friendly sustainable agricultural practices. Bio-fertilizer is defined as a substance which contains living organisms which, when applied to seed, plant surface, or soil, colonize the rhizsphere or the interior of plant the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey 2003). Biofertilizers are well recognized as an important component of integrated plant nutrient management for sustainable agriculture and hold a great promise to improve crop yield (Narula *et al.* 2005; Wu *et al.* 2005). A group of bio-fertilizers contain termed plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.* 1980) and among them are strains from genera such as *Pseudomonas*, *Azospirillium*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Erwinia* and *Flavobacterium* (Rodriguez and Fraga 1999). Thus organisms are important for agriculture in order to promote the circulation of plant nutrients and reduce the need from chemical fertilizers. Most of the studies reporting beneficial effects of the above mentioned PGPR were carried out in warm and subtropical climates with favorable ambient temperatures. These bacteria may not be effective in cold temperature conditions. Therefore, a study was conducted in order to investigate the effects of alone and in combinations with N_2-fixing and P-solubilising PGPR strains on nodulation, plant growth, nutrient uptake and grain yield of wheat in the cold highland (Erzurum) and low land (İspir) plateaus.

Material and methods

Site selection

In order to investigate the effects of seed inoculation with PGPR on yield and yield components of wheat *(Triticum aestivum spp. vulgare var.* Kirik*)* in the field experiments at two sites, 150 km apart from each other. The first (I) field is located in the Coruh valley in Erzurum in eastern Anatolia, 40° 28' N and 40° 58' E with at an altitude of 1120 m, and the second field is in the Erzurum Experimental Farm of the Atatürk Üniversity and in Erzurum in Eastern Anatolia, 29° 55' N and 41° 16' E with an altitude of 1950 m. The soils were classified as Entisol and Aridisols according to the USDA taxonomy (Soil Survey Staff 1992).

Field experiment
The experiments were conducted using a randomized complete block design in a factorial arrangement each having 10 main treatments as control (without inoculation and any fertilizer application), Nitrogen (80 kg N/ha), Nitrogen (40 kg N/ha), *Bacillus* OSU-142, B*acillus* M-3, *Azospirillum* sp. 245, Mixed (OSU-142 + M-13+ *Azospirillum* sp. 245), *Bacillus megaterium* RC07, *Paenibacillus polymyxa RC05* and *Raoultella terrigena* for 2007 and 7 treatment also added in 2008, FS Tur, OSU142 AMP Res, M-3 Amp Res, sp.245 Amp Res, *P. polymyxa* 2/2, *B. megaterium* T17, and Mixed + 40 kg N/ha. Wheat was sown in 7 m x 4 m plots having 34 rows so as to give 18 kg seeds da^{-1} (430 seeds per m^2) on 10 and 17 May in 2007, 4 and 2 May in 2008 at site I and site II. Maximum care has been taken not to contaminate and mix bacterial inoculations during sowing.

Statistical analysis
The data were subjected to analysis of variance (ANOVA) using SPSS 13.0 (SPSS Inc. 2004) statistical program. Since "year x treatment" interaction was not significant in many parameters evaluated, data were combined over years, and means were presented. Mean values were separated according to LSD test at P=0.05.

Result and Discussion
Yield and yield parameters
Bacterial inoculations improved the wheat growth and growth parameters. The performance of the plants was better in inoculated treatments in comparison to the control. The results showed that grain yield (GY), straw yield (SY), harvest index (HI) and total yield (TY) of wheat cultivars in each of two locations in both years significantly increased by N_2-fixing and P-solubilizing PGPR strains application compared with the control. The lowest GY, SY, TY and HI were recorded in the control treatment and the bacterial inoculations increased GY by 8.6-43.7%, SY by 1.82.-12.9%, TY by 1.93- 19.80% and HI by 1.33-45.7% over the control on 2-year average at two locations, respectively (Table 1, 2). The GY of plant in 2007 was higher than GY of 2008, but the highest TY of wheat was found in 2008 at each location (Table 1, 2). The highest GY (3.68-2.88 Mg/ha), SY (9.73-8.57 Mg/ha), and TY (13.41-11.45 Mg/ha) in both years average at two locations were obtained from 80 kg N/ha , and followed by in combination (OSU-142 + M-13+ *Azospirillum* sp. 245) treatment at 3.47-2.87 Mg/ha for GY, 8.73-6.75 Mg/ha for straw and 12.20-9.65 Mg/ha for total yields, respectively. In other words, mixed PGPR inoculations with the strain of OSU-142 + M-13 + *Azospirillum* sp.245 has significantly increased GY, SY, TY and HI of wheat as good as full doses of nitrogen. When it is compared to 40 kg N/ha treatment, bio-fertilizer applicants were more effective to increase the grain yield. On the other hand harvest index (HI) of two years average in two locations was better in inoculation treatments in comparison to the control and mineral fertilizer (80 and 40 kg N/ha) treatments. While the lowest HI values were recorded in the control treatment, the highest value was obtained from the OSU-142 + M-13+ *Azospirillum* sp. 245 and B*acillus* M-3 treatments. In the current system, the results support reduced fertilizer rates down to 50% if PGPR was added because that is the minimum at which results were consistent. This is different from the observations of Canbolat *et al.* (2006) and Elkoca *et al.(*2008), who reported no significant difference in root and shoot biomass of barley or seed yield and biomass of roots and shoots of chickpea, respectively, when inoculant alone or fertilizer alone was used.

Effects of bio-fertilizer on plant nutrient element (PNE) contents of different parts of the plant
N_2-fixing and P-solubilizing PGPR strains application promoted PNE contents of different parts of the plant. Although the highest leaf, grain, and straw N contents were obtained from mixed inoculation with the OSU-142 + M-13 + *Azospirillum* sp.245 +40 kg N/ha, which increased N contents of leaf, grain and straw of plant by 52.6%, 83.4%, and 83.0%, respectively, compared with the control treatment. While S contents were obtained from treatment OSU-142 + M-13 + *Azospirillum* sp.245and increasing rate were 64.9% for grain and 65.0% for straw. P, K, Mn, Fe, and Zn contents were obtained from B*acillus* M-3 treatment, and increase rate for leaf, grain and straw of plant were 42.7%, 50.6%, 82.5% for P, 26.3%, 112.0%, 110.0% for K, 49.1%, 121.2%, 120.0% for Mn, 90.1%, 102.6%, 105.2% for Fe, and 49.2%, 75.4%, 75.4% for Zn. Some of the previous studies with the same PGPR strains tested on chickpea, barley, raspberry, apricot and sweet cherry have been reported similar findings confirming our data in the present work. The use of the OSU-142 and M-3 in chickpea (Elkoca *et al.* 2008), barley (Cakmakci *et al.* 2007), raspberry (Orhan *et al.* 2006), apricot (Esitken *et al.* 2003), sweet cherry (Esitken *et al.* 2006) and strawberry (Güneş *et al.* 2009) stimulated macro- and micro-nutrient uptake such as N, P, K, Ca, Mg, Fe, Mn, Zn, Cu.

Table 1. Yield and yield components of wheat plant growth at two locations in 2007 (t/ha)

Inoculant	I. Field			II. Field		
	Grain	Straw	Total biomass	Grain	Straw	Total biomass
Control	3.34 d	6.57 e	9.90 d	2.46 d	8.90 c	11.35 c
Nitrogen (80 kg N/ha)	4.02 a	8.82a	12.83 a	3.34 a	10.66a	14.00 a
Nitrogen (40 kg N/ha)	3.79 a-c	7.87b	11.66 ab	2.27 ab	11.07a	13.33 ab
Bacillus OSU-142	3.69 a-d	7.47 bc	11.15 bc	3.02 a-c	9.04 b	12.06 bc
B*acillus* M-3	3.50 b-d	6.69e	10.18 d	2.85 c	7.98 d	10.83 c
Azospirillum sp.245	3.76 a-c	8.07a	11.83 a	3.04 a-c	9.18 b	12.22 bc
OSU-142 + M3+ Az.245	3.85 ab	8.04a	11.89 a	3.10 a-c	9.41 b	12.51a-c
B. megaterium RC07	3.43 cd	7.39 cd	10.83 c	2.87 c	8.26 c	11.13 c
P. polymyxa RC05	3.72 a-c	7.52 bc	11.24 bc	2.93 bc	8.69 c	11.61 bc
B. licheniformis RC08	3.46 cd	6.65e	10.11 d	2.72 d	8.56 c	11.28 c

Table 2. Yield and yield components of wheat plant growth of two locations in 2008 (t/ha)

Inoculants	I. Field			II. Field		
	Grain	Straw	Total biomass	Grain	Straw	Total biomass
Control (without inoculation and fertilizer)	2.79 c	7.79d	10.58 c	1.20 g	7.11b	8.31abc
Nitrogen (80 kg N/ha)	4.18 a	8.90 a	13.08a	1.95 a	8.51a	10.46 a
Nitrogen (40 kg N/ha)	3.57 a-c	7.46d	11.03bc	1.58 cd	8.25a	9.83 ab
Bacillus OSU-142	3.35 a-c	8.37 a-c	11.72 a-c	1.53 cde	6.18bc	7.72 bcd
Bacillus M-3	3.65 a-c	7.03d	10.68 c	1.50 cde	5.51 d	7.01 cd
Azospirillum sp.245	4.17 a	7.78d	11.95 a-c	1.39 ef	4.70 e	6.09 d
OSU-142 + M-13+ *Azospirillum* sp.245	3.94 ab	8.68 ab	12.62ab	1.80 b	4.83 e	6.63 cd
Bacillus megaterium RC07	3.28 a-c	8.41 bc	11.69a-c	1.66 bc	6.65 bc	8.31 abcd
Paenibacillus polymyxa RC05	3.81a-c	8.02 bc	11.83a-c	1.39 ef	6.39 bc	7.77 bcd
Raoultella terrigena	3.59 a-c	7.37d	10.96bc	1.39 ef	5.71 d	7.09 cd
FS Tur	2.99 bc	7.47d	10.46 c	1.59 c	7.35b	8.94 abc
OSU142 AMP Res	3.67 a-c	8.23 a-c	11.90a-c	1.49 cde	6.27bc	7.77 bcd
M-3 Amp Res	3.54 a-c	8.19 a-c	11.73 a-c	1.53 cde	6.53 bc	8.06bcd
Sp.245 Amp Res	3.20 a-c	8.52 a-c	11.72 a-c	1.42 def	8.49a	9.90 ab
P. polymyxa 2/2	3.20 a-c	8.92ab	12.12 a-c	1.39 ef	6.25bc	7.64 bcd
B. megaterium T17	3.41 a-c	8.42 a-c	11.83 a-c	1.30 fg	6.63bc	7.93 bcd
OSU-142 + M-13 + *Azospirillum* sp.245 +40 kg N/ha	3.83 a-c	8.92a	12.75a	1.66 bc	4.47 e	6.12 d

Conclusions

Our results indicated that microbial inoculation of seeds with N_2-fixing and P-solubilizing PGPR strains alone and in combination, may substitute costly NP fertilizer in wheat production even in cold highland and low land areas. In view of environmental pollution in case of excessive use of fertilizers and due to high costs in the production of N and P fertilizers, bacteria tested in our study may well be suited alone or in combination to achieve sustainable and ecological agricultural production in the region. An important nutritional problem of developing countries is micro-nutrient malnutrition, also called hidden hunger. Our results also indicated that alone or in combination inoculations with N_2-fixing and P-solubilising PGPR strains could increase mineral concentration and accumulations in the grain. This paper supports the view that inoculations with PGPR have some potential to serve as a means to reduce hidden hunger trough enhanced mineral concentration and accumulation in grain.

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The Effects of Plant Growth Promoting Rhizobacteria on Healthy Plant Growth of Tomato Affected by Soil Sickness.

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Abstract
The use of antagonistic microorganism for the biological control of root disease is becoming an important alternative or supplement to chemical pesticides. In the present study, we investigated the potential of beneficial rhizobacteria to interact synergistically with indigenous arbuscular mycorrhiza fungi (AMF), furthermore for improvement plant growth of two tomato varieties using replant disease. Soil inoculation with *Pseudomonas* sp. "Proradix®" and *Bacillus amyloliquefaciens* FZB42 significantly improved the root and shoot biomass production of the two tomato varieties growing on pathogen-infected soil. Roots of both tomato varieties were not only healthier but also showed a significantly higher colonization by AMF. Root lesions was significantly lower in the rhizosphere soils of both tomato varieties when inoculated with *P.* sp. "Proradix®" or *B. amyloliquefaciens* FZB42 compared to the untreated control. The concentration of macro and micronutrients in tomato shoots was higher in the *P.* sp. "Proradix®" and *B. amyloliquefaciens* FZB42 treated plants when compared to the untreated control. The result obtained suggest an important role of rhizosphere interactions for the expression of bio-control mechanisms by inoculation with effective *Pseudomonas* and *Bacillus* strains independent of simple antagonistic effects.

Key Words
Plant Growth-Promoting Rhizobacteria (PGPR), arbuscular mycorrhiza fungi (AMF), soil sickness, Tomato.

Introduction
Establishing of the same crops in long term at the same site can cause a problem known as replant disease (Utkhede 2006). Mono-cropping systems lead to decreases in the abundance of beneficial microorganisms such as *Pseudomonas* and to increases in the population of soilborne pathogens in the soil (Joshua-Otieno and Jingguan 2006). Soilborne plant pathogen control by fumigation, chemical pesticide and soil solarization is intensively investigated. However, research is needed to develop cultural and biological control methods to induce resistance of host plant. The use of plant growth promoting rhizobacteria (PGPR) to protect crops from soilborne disease eventually inducing healthy growth of plants with high yields at lower cost and minimum risk to humans and environment is a promising strategy. In the present study, we conducted a pot experiment to investigate the potential of beneficial rhizobacteria (*Pseudomonas* sp. "Proradix®" (DSMZ 13134) (Proradix®, Sourcon Padena, Tübingen-Germany) and *Bacillus amyloliquefaciens* FZB42 (RhizoVital®, ABiTEP, Berlin, Germany) to interact synergistically with indigenous, site specific and adapted AMF. It was hypothesized that these rhizobacteria improve indigenous AMF infection, nutrient acquisition and growth of two tomato varieties, and suppression of soilborne pathogens under high pathogen pressure induced by replant disease soil from the rooting zone of a tomato plantation with known replant disease problems.

Methods
Plant and microbial inoculums' preparation
Tomato seeds (*Lycopersicon esculentum* Mill., varieties Money Maker and Hellfrucht Hillmar) were surface sterilized by first shaking them in a 75% Ethanol solution for 1 min and then in a 1.5% Sodium hypochloride (NaOCl) solution for 3 min. First, the tomato seeds were cultivated in pots containing 50 g substrate (Einheitserde Type P, Einheitserde und Humuswerke Gebr. Patzer, Sinntal-Jossa, Germany). After two weeks, the seedlings were transplanted to pots containing 1 kg replant disease soils/sand mixture (3:1). Before, the plants were treated by dipping of the roots into preparations of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (Proradix®, Sourcon Padena, Tübingen-Germany) (1.5×10^{10} cfu/l sterile distilled water) and *Bacillus amyloliquefaciens* FZB42 (RhizoVital®, ABiTEP, Berlin, Germany) (100 g/l sterile distilled water). The non-treated control plants were dipped into pure sterilized distilled water. Before planting the soil was fertilized with 100 mg N, 50 mg P, 150 mg K, 50 mg Mg, 0.06 mg Fe per kg replant disease soils. The

replant disease soil was collected from the root zone of tomato field at the horticultural experimental station of the Universität Hohenheim (Stuttgart-Germany) with known replant disease problems. Before use, the soil was passed through a 2 mm sieve, moistened to about field capacity and stored in a closed plastic box for about one week. The pots were arranged in a completely randomized design pattern in the greenhouse. A heating-cooling system adjusted the soil temperature conditions to a day/night cycle: 14 h at 25°C/10 h at 19°C. The relative humidity in the greenhouse was about 75%. The plants were irrigated when required and harvested four weeks after transplanting.

Plant harvest, nutrient concentration analysis, mycorrhizal root colonization and root lesions

At harvest, roots were thoroughly washed and blotted. A subsample was taken for the assessment of mycorrhiza formation. Shoots and roots were dried 72 hours at 65°C for dry weights were determined. Mineral elements were determined by atomic absorption spectrophotometry (Mn, Zn and Cu), flame-photometry (Mg and K) and photo-spectrophotometry (P) after wet digestion. Assessment of mycorrhizal root colonization was based on Koske and Gemma (1989) and Kormanik and McGraw (1984) and root lesion was based on Tennant (1975).

Statistical analysis

The experimental design was a completely randomized 2 (tomato varieties) x 3 (PGPR) factorial design with 4 replicates. Data of relative percentage of mycorrhiza infection and root lesions were normalized by arcsin√% transformation before being subjected to a two way analysis of variance (ANOVA) (Gomez and Gomez 1984). A Tukey test at a significance level of $P<0,05$ was conducted on the transformed data after the ANOVA to distinguish between differences among the treatments. The results in tables, text and figures are given as means. All statistical analyses were performed using Sigma Stat version 2.03 statistical software (SPSS Inc. Chicago. IL. USA).

Results and Discussion

In general, Soil inoculation with *Pseudomonas* sp. ‘‘Proradix®’’ (DSMZ 13134) (Proradix®, Sourcon Padena, Tübingen-Germany) and *Bacillus amyloliquefaciens* FZB42 (RhizoVital®, ABiTEP, Berlin, Germany) significantly increased the root and shoot biomass production of the two tomato varieties growing on pathogen-infected soil (Figure 1). Roots of both tomato varieties were not only healthier but also showed a significantly higher colonization by AMF and lower root lesions caused by soilborne pathogens (Figure 2). The percentage of AMF infected roots was significantly higher in the tomato variety Hellfrucht Hillmar than in the variety Money Maker.

The concentration of macro and micronutrients in tomato shoots was higher in the *P.* sp. ”Proradix®“ and *B. amyloliquefaciens* FZB42 treated plants when compared to the untreated control (Table 1). This is in accordance with authors (Siddique *et al.* 2001; Barea *et al.* 2002; Akkopru and Demir 2005; Yusran *et al.* 2008) who reported that AMF colonization in the roots of many crops greatly enhanced the uptake of phosphorus and micro nutrients and reduce severity of plant diseases. This indicates that the AMF infection potential in the soils was not generally low but rather suppressed directly by pathogens or indirectly as consequence of poor root development.

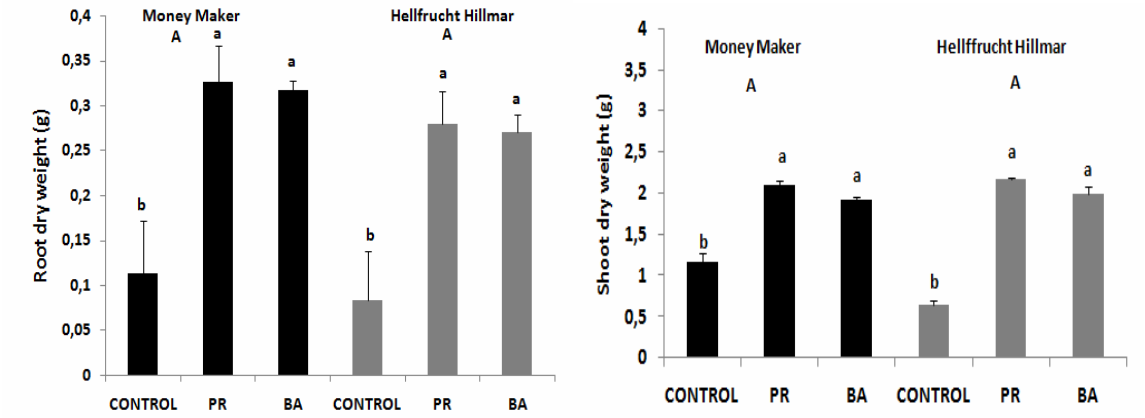

Figure 1. Root dry weight (left) and shoot dry weight (right) of two tomato varieties 6 weeks after planting with *Pseudomonas* sp. ‘‘Proradix®’’ (PR), *Bacillus amyloliquefaciens* FZB42 (BA) on replant disease soil. Vertical bars indicate standard errors of the mean (n=4). Different small letter above the bars indicate significant differences between the treatments within one tomato variety and capital letter indicate significant differences between the two tomato varieties (Tukey test, p<0,05).

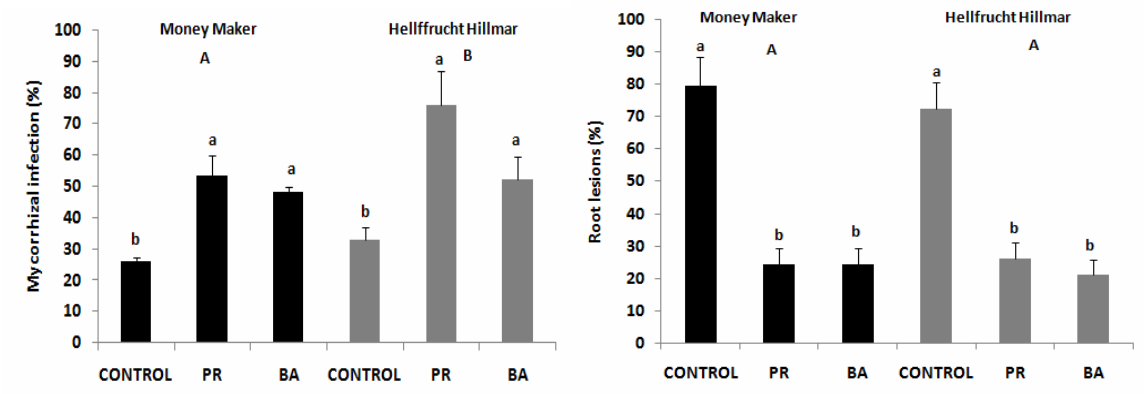

Figure 2. Percentage of roots infected by indigenous AMF (left) and root lesions (right) 6 weeks after planting with *Pseudomonas* sp. ''Proradix®'' (PR) or *Bacillus amyloliquefaciens* FZB42 (BA) on replant disease soil. Vertical bars indicate standard errors of the mean (n=4). Different small letters above the bars indicate significant differences between the treatments within one tomato variety and different capital letters indicate significant differences between the two tomato varieties (Tukey test, p<0,05).

Table 1. Macro- and micronutrient concentration in the shoot dry matter of two tomato varieties six weeks after planting with *Pseudomonas* sp. ''Proradix®'' (PR) or *Bacillus amyloliquefaciens* FZB42 (BA) in replant disease soil. Different letters in the same row indicate significant differences between all treatments within each variety and asterisk following nutrient symbol indicate significant differences between two variety(Tukey test, p<0,05). NS = No significant difference.

Nutrient concentration in the shoots	Tomato variety					
	Money Maker			Hellffrucht Hillmar		
	CONTROL	PR	BA	CONTROL	PR	BA
P (mg/g)*	2,4 b	3,5 a	3,2 a	1,6 c	3,7 a	2,9 b
K (mg/g)*	21,4 b	33,6 a	29,7 a	18,9 b	24,8 a	24,4 a
Mg (mg/g) ns	2,8 b	4,0 a	3,8 a	3,0 b	3,7 a	3,5 a
Mn(mg/kg)*	7,8 c	27,5 a	14,9 b	5,3 c	20,8 a	18,9 b
Zn (mg/kg)*	7,3 c	32,5 a	16,8 b	4,5 c	11,9 a	9,8 b
Cu(mg/kg)*	3,7 c	5,6 a	5,1 b	4,5 c	9,5 a	7,7 b

Conclusion

In conclusion, the result obtained suggest an important role of rhizosphere interactions for the expression of bio-control mechanisms by inoculation with effective *Pseudomonas* and *Bacillus* strains in addition to simple antagonistic effects. The use of PGPR may provide immediate benefits as improving mycorrhization, supporting healthy growth of plants and suppressing soilborne pathogens. However, further field based research is necessary to ensure a high efficiency and reliability of those products under field conditions.

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The role of mycorrhizas in plant nutrition: field and mutant based approaches.

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Abstract
The majority of plants, including most crops, form arbuscular mycorrhizas (AM). These associations play an important role in the growth and nutrition of plants. Here we present results from our ongoing research on the role of AM in sustainable production systems. Firstly, we present results of a survey of the formation of AM by field grown tomato plants from across the south-eastern Australian processing tomato industry. This survey revealed low levels of colonisation, which could be explained by various farm management and edaphic factors. Secondly, to explore the role of AM in these farming systems, we grew a mycorrhiza defective tomato mutant and its mycorrhizal wild type progenitor in in-tact cores containing tomato farm soils. This novel genotypic approach to controlling for AM colonization allows us to study the formation and functioning of AM in field soils without the need to fumigate soils to establish non-mycorrhizal controls. Using this approach we found an important role for AM in the nutrition of tomato plants. Given the role of AM in plant nutrition, and the low levels of colonization in the survey, our ongoing research aims to identify better ways to manage AM in the field.

Key Words
Mycorrhizas, tomato, plant mutant, plant nutrition.

Introduction
Arbuscular mycorrhizas (AM) are associations formed between the roots of most terrestrial plant species and a group of specialized soil fungi (Smith and Read 2008). The formation of AM has a significant impact upon plant growth and nutrition, with bi-directional exchange of inorganic nutrients (P, Zn and others) and carbon between the plants and fungi (Cavagnaro 2008; Marschner and Dell 1994). It has been demonstrated that the plants can receive up to 100% of their P via the mycorrhizal pathway (Smith *et al.* 2004), and 4-20% of a plants C can be transferred to the fungi (Cavagnaro *et al.* 2008; Jakobsen and Rosendahl 1990). This transfer of resources between the plants and fungi have profound effects on plant growth, nutrition and ecology, and have been the focus of considerable interest (Smith and Read 2008). However, if we are to capitalize on the potential benefits to agriculture of forming AM, it is essential that the formation and functioning of these associations be studied in a field setting. Here we present results of field and glasshouse based studies of AM in tomato framing systems.

AM have an important role to play in increasing the sustainability of agricultural systems (Cardoso and Kuyper 2006). However, few studies have directly assessed the role of AM under field conditions. This can in part be attributed to difficulties associated with the establishment of non-mycorhrizal controls in field soils. For example, fumigation of soils can eliminate other members of the soil biota (many of which are involved in nutrient cycling) as well as affect crop growth and nutrition through other mechanisms (Cavagnaro *et al.* 2006; Ryan and Angus 2003). We have overcome this issue using a novel genotypic approach to establishing non-mycorrhizal controls (Cavagnaro and Jackson 2007; Cavagnaro *et al.* 2007; Cavagnaro *et al.* 2006; Cavagnaro *et al.* 2008; Cavagnaro *et al.* 2004). By growing a mycorrhiza defective tomato mutant (named *rmc*) (Barker *et al.* 1998) and its mycorrhizal wild-type progenitor in field soils, it has been possible to explore mycorrhizal functioning, in particular nutrient interception, without the need to fumigate soils, thereby leaving the wider soil biota intact.

The overarching goal of this research is to understand the formation and functioning of AM in the field. This information will be essential in developing farming systems that promote AM and their potential benefits. Here we present results from selected studies which explore different aspects of this goal.

1. Formation of AM in the field:
To assess the formation and functioning of AM in the field, we undertook a survey of AM formed by

tomatoes in southeastern Australia. This survey sought to determine the levels of AM colonization on processing tomato farms, and identify factors impacting levels of colonization.

2. Nutrient uptake and interception by AM:
AM play an important role in plant nutrient uptake. This is important with respect to both plant nutrition and minimizing the loss of nutrients from soils via leaching. Using our genotypic approach for controlling AM colonization, we have studied nutrient uptake by AM in the field, and in field collected soils. Here we present results of selected studies to illustrate the role of AM in nutrient uptake by tomato plants.

Methods

1. Formation of AM in the field

Soils and roots were collected from 17 tomato farms from across northern Victoria, Australia. Samples were collected as fruits were maturing, in January-February, 2009. Briefly, soils were collected from immediately between tomato plants growing in beds. Roots were washed from the soil with reverse osmosis water, cleared with KOH and stained with ink and vinegar using a modification of the method of Vierheilig *et al.* (1998). Colonization of roots was determined using a modification of the methods of McGonigle *et al.* (1990). Soils were also analysed for a wide range of key physicochemical properties.

2. Nutrient uptake and interception by AM

To assess the functioning of AM we have grown the mycorrhiza defective tomato mutant (*rmc* hereafter), and its wild type progenitor *Solanum lycopersicum* L. cv. 76R (see Barker *et al.* 1998) (76R, hereafter) in a range of field soils. In this study, soil was collected from tomato fields using in-tact cores (0-15 cm depth). The cores were returned to the laboratory and seeds of either the *rmc* or 76R genotypes of tomato planted in them. The plants were grown in a glasshouse and harvested after 2 months. Mycorrhizal colonization of roots was assessed (as above) and plant growth and nutrient contents and concentrations (via ICP-AES) were measured (Cavagnaro *et al.* 2006). Data from one site are presented in this paper.

Results and Discussion

1. Formation of AM in the field

In our detailed survey of mycorrhizal colonization of roots from across the processing tomato sector of southeastern Australia, we found that mycorrhizal colonization of roots was generally low. Indeed, ca. 40% of root samples contained no colonization at all. This indicates that the potential benefits of forming AM (i.e. nutritional and biomass) are not being realized in these tomato fields. Furthermore, where roots were colonized, the mean level of colonization across all sites was very low (ca. 5% of root length). While these levels are low, they are in line with a previous study focusing on field grown tomatoes in a sourthern Florida research station (Rasmann *et al.* 2009), but not in organically managed tomato farms in California (Cavagnaro *et al.* 2006). Analysis of farm management records revealed that AM colonization was not present predominantly in fields where soils had been fumigated at the start of the growing season. Moreover, there was a ca. five fold reduction in colonization of roots where plants were grown in soils that had been fumigated. While not necessarily unexpected, this highlights the importance of farm management on AM. If we are to capitalize on the benefits for forming AM, the impact of soil fumigation needs to be carefully considered. Tomatoes are grown in nurseries prior to translating in the field. Our ongoing research is investigating the potential to pre-inoculate seedlings with AM fungi in the nursery phase of production to improve levels of AM colonization in the field.

In addition to the influence of soil fumigation on AM we also explored the relationship between AM colonization (where it was present) and a wide range of edaphic factors. While colonisation was correlated with soil N nutrition, it was not related to soil P nutrition, as in previous studies (Smith and Read 2008). Together these data highlight the need to consider AM in the management of agricultural soils. To this end, the relationship between the formation of AM, soil physicochemical properties and other edaphic factors in this field survey is currently being assessed in detail.

2. Nutrient uptake and interception by AM

When grown in in-tact cores containing tomato farm soil, the mycorrhiza defective tomato mutant (*rmc*) had essentially no colonisation (less than 2%). Conversely, the mycorrhizal tomato genotype was well colonized for tomato in field collected soils (>15%). This differential colonisation of the two genotypes is consistent with our earlier studies(Barker *et al.* 1998; Cavagnaro *et al.* 2007; Cavagnaro *et al.* 2006; Cavagnaro *et al.*

2008; Cavagnaro *et al.* 2004), and thus, the two genotypes provided mycorrhizal and non-mycorrhizal plants for comparing the functioning of AM in a field soil.

While the two genotypes were differentially colonised, there was little difference in the total biomass of the genotypes. Specifically, growth of the tomato plants was marginally higher (P=0.07) in the mycorrhizal (3.2+/-0.3 g dry weight) than the non mycorrhizal plants (2.2+/-0.4 g dry weight). This finding is in agreement with earlier studies using a range of field soils in both the field and glasshouse (Cavagnaro *et al.* 2006; Cavagnaro *et al.* 2008; Cavagnaro *et al.* 2004; Poulsen *et al.* 2005). This "matched growth" of the genotypes represents a situation where the cost (C for the fungus) and benefit (nutrients to plant, see below) of forming the AM are equivalent (Johnson *et al.* 1997). It is important to note, however, that in most studies biomass is quantified before the plants have reached maturity. Thus, the lack of a vegetative growth response does not necessarily preclude a mycorrhizal "benefit" at a later stage of development. This is an important focus of our ongoing research.

In this study there was no difference in the P concentration of the genotypes (data not shown), in contrast to our earlier studies (Cavagnaro *et al.* 2006; Cavagnaro *et al.* 2008). There was, however, a significant difference in whole plant Zn content. Specifically, the whole plant Zn content (roots+ shoots) of the non-mycorrhizal genotype (77+/-15 μg/plant) was significantly (P<0.001) lower than that of than in the mycorrhizal plants (243+/-18 μg/plant). This considerable increase in Zn content of the mycorrhizal plants highlights the role of AM in plant nutrition generally, and the interesting role that AM play in plant Zn nutrition (Cavagnaro 2008).

Conclusion

The findings reported here, and in our past and ongoing research, indicate that AM have an important role to play in the growth and nutrition of tomatoes. The results of our sector wide field survey indicate that these potential benefits may not be being fully realized. This presents an important challenge and opportunity to capatalize on the benefits that forming AM. Improvements in the formation of AM in the field must, however, be predicated upon a sound understanding of the impacts of AM on plant growth, nutrition and physiology. Our research seeks to address this knowledge gap by using a novel genotypic approach to address a long standing challenge in the study of AM in "real world" soils.

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The spatial structure of soil microbial properties in an upland grassland

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Abstract
We characterised the spatial structure of soil microbial properties (microbial biomass-C, microbial biomass-N, dissolved organic carbon (DOC), dissolved organic nitrogen (DON), dissolved inorganic nitrogen (DIN), soil respiration and net nitrogen mineralization, metabolic quotient (qCO_2) and C/N ratio in the microbial biomass) in an unimproved upland grassland in the Yorkshire Dales, United Kingdom. The grassland was selected because it had been ungrazed for thirty years. A 20 m x 20 m plot was marked and 100 soil samples were taken on a regular grid of 2 m x 2 m. The botanical composition of the vegetation associated with each soil sample was also determined. Both, descriptive statistics parameters and semivariogram analysis were considered for assessing data variability. Soil properties showed very high variability (CV from 50% to 84%). Total plant biomass was rather homogeneous (CV=8%), however, individual plant species distribution was highly skewed. Correlations between soil properties were generally strong. Soil respiration, net N-mineralization and DIN did not exhibit spatial dependence at the sampled scale, indicating that data for these variables collected at 2m distance are independent. The spatial dependence of the remaining soil properties was described by a nugget component plus a structure, where the proportion of structural variance was highest for DOC and DON. Also the spatial dependence of plant biomass was either absent or scarce. In cases where spatial dependence was found, ranges extended from approximately 3.2 to 8 m, dispersed throughout both soil properties and vegetation; places further apart than this are spatially independent. Furthermore, mapping of the soil properties and vegetation allowed description and interpretation of interaction between them. Our data demonstrate high spatial variation in microbial properties in upland grasslands, and suggests plant cover may influence the different soil microbial properties.

Key Words
Microbial community, spatial variability, grasslands, geostatistics, microbial biomass, soil respiration

Introduction

Large-scale determinants of soil microbial properties are relatively well known, with climate, soil type, land-use, management practices and topographic feature being among the most influential factors. At smaller scales, however, there is much less agreement about the environmental factors that drive soil microbial communities and the processes that they regulate. On the other hand, although the mechanisms generating plant spatial patterns have been widely studied (Batllori *et al.* 2009), few studies concentrated on the consequences of these patterns on belowground soil properties. Thus, at present, community level associations between vegetation and microbial assemblages are not well understood. There is stronger evidence that there may be characteristic microbial communities associated with particular plant species (Bardgett *et al.* 1998). The mechanisms by which such associations are generated are generally supposed to be related to the quantity and quality of substrates deposited below-ground by plants, in the form of root and shoot litter and root exudates. This means that there would be, at least to a certain extent, spatial coupling between plant and microbial communities (Grayston *et al.* 1998).

In ecosystems where plants tend to be sparse, there is evidence that soil organisms are affected by proximity to individual plants. In grasslands, plant density is very high and there is much less information available about the spatial organisation of microbes relative to plant types. Previous studies on temperate upland grasslands have shown some evidence for community-level coupling (Clegg *et al.* 1999). However, such relationships were obscured by high levels of spatial variation as the composition of communities a few metres apart within a grassland can be as different as those separated by hundreds of kilometres (Clegg *et al.* 1999). One approach to explore drivers between soil microbial community and plant community would be to determine statistically natural variation in soil microbial community and analyse associations with plant community.

In the present study, the spatial properties of soil microbial community structure, and associated vegetation, were measured in an unimproved upland grassland. The grassland had not been grazed for the last 30 years, so plant growth and community structure are mostly reliant upon natural processes of nutrient cycling and

abiotic environmental conditions. Since the underlying soil type and micrometeorology were effectively constant between samples, such factors would not confound the detection of any vegetation-microbe associations that may occur in multiple-site studies. Thus, the aim of this study was to characterize the spatial variability and distribution of soil biological properties and try to relate them with the variability of plant vegetation.

Methods

The study area is located in the south-western corner of the Yorkshire Dales, on top of Ingleborough (United Kingdom) 54°9′ N 2°23′ W and 700 m.a.s.l. The soil is located in an acid bog (45 % average organic carbon, pH=3.5) and the parent material is limestone. A 10x10 m area of ostensibly uniform grassland was randomly selected within the field site, avoiding evident major topological variation (emergent rock, erosion points, slopes, etc.). Soil and plant were sampled every two meters, using a regular grid (thus, the total number of samples was 100). At each sampling point, an intact sample of soil (5 cm diameter, 15 cm deep) was taken using a core auger. Vegetation was sampled (hand removed) in a 30x30 cm area around the soil sampling point. Sampling took place at the end of June 2009. Microbial biomass C and N were measured using the fumigation–extraction techniques of Vance *et al*. (1987). Briefly, soil samples (5 g) were fumigated with $CHCl_3$ for 24 h at 25º C. After the removal of the $CHCl_3$, soluble C was extracted from the fumigated and from unfumigated samples with 0.5M K_2SO_4 for 30 minutes on an orbital shaker. Total organic C (TOC) in filtered extracts (Whatman No. 1) was determined using a Shimadzu 5000A TOC analyser. Microbial C flush (difference between extractable C from fumigated and unfumigated samples) was converted to microbial biomass C using a k_{EC} factor of 0.35 (Sparling *et al.* 1990). Extractable N in the above extracts was determined by oxidation with $K_2S_2O_8$ using the methodology of Ross (1992), and measurement of the resultant NO_3-N and NH_4-N by auto-analyser procedures using a Bran-Luebbe Autoanalyser. The microbial N flush was converted to microbial biomass N using a k_{EN} factor of 0.54 (Brookes *et al.* 1985).
Microbial activity was measured as basal respiration, using the method described by Bardgett et al. (1997). The pots were placed in Kilner jars and incubated for 24 h at 25ºC. One millilitre headspace gas was then removed and CO_2 concentration was measured against a 1% standard gas on an infra-red gas analyser and expressed as ml CO_2 g^{-1} dry soil hr^{-1}. Dissolved organic carbon (DOC), dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN) were measured by adding 35 ml distilled water to 5 g moist soil samples, which were shaken on an orbital shaker for 10 min prior to being filtered through Whatman No. 1 paper. Total and inorganic C in the extract were determined using a Shimadzu 5000A TOC analyser, and DOC was then calculated by subtracting the amount of inorganic C from the total C in the samples. Total N was determined by oxidation with $K_2S_2O_8$ using the methodology of Ross (1992), and measurement of the resultant NO_3-N and NH_4-N by auto-analyzer procedures using a Bran-Luebbe Autoanalyzer. DON was calculated by subtracting the amount of inorganic N from the total N in the samples.

To determine net N mineralisation, 10 g soil samples were extracted for 30 min with 50 ml of 1 M KCl before and after incubation for 14 days at 25°C at field moisture content. Total inorganic N was determined in the extracts using an autoanalyzer. Net nitrogen mineralisation (mg kg^{-1} day^{-1}) was calculated as the difference between the values obtained before and after incubation. At each sampling points, plant samples were classified according to their species, air dried for 48 hours (70ºC) and weighted. Eight plant species were detected at the study site, belonging to the following genera: *Lycopodium, Racomitrium, Sphagnum, Deschampsia, Agrostis, Eriophorum, Vaccinium* and *Carex* and were present, respectively, in the following amounts in the studied area: 10.82, 18.29, 4.70, 13.62, 37.90, 0.96, 10.92 and 2.97 g m^{-2}.
The patterns of spatial variability of the studied soil properties and plant biomass data sets were assessed by univariate geoestatistics. Semivariograms were computed and models were fitted for each study soil and plant variable. Model adjustment was performed by visual inspection and statistical fitting Maps were obtained by two methods: inverse distance interpolation and, if spatial dependence was evidenced, also by kriging interpolation.

Results

A consistent feature of many of the soil properties measured in this study was a large range in the magnitude of the parameters, exhibiting high levels of soil spatial heterogeneity. As an example, soil respiration showed a 40-fold variation between its maximum and its minimum value varied. This variation was also very high for DIN (23-fold) and for DOC (11-times). Coefficients of variation of soil properties ranged between 50% and 84%. Variation was also very high in the plant cover, with coefficients of variation always higher than 78 % and as high as 300 % for *Eriophorum* (see Table 1). Plant biomass distribution of each individual

species was strongly skewed, because most of them showed areas without some of the plant species. In spite of the variation in the individual plants, the total weight of the plant cover was quite homogeneous (minimum value of 129 g m^{-2} and maximum value of 176 g m^{-2} and CV was 8%). Pearson correlation coefficients among most of the soil properties measured were high (data not shown).

Table 1. Statistical summary for all properties measured.

	Minimum	Maximum	Average	Median	Standard deviation	CV	Skewness	Kurtosis
Biomass C (mg kg^{-1})	420	2762	1292	1115	710	55	0.433	-1.114
Respiration(ml CO_2 g^{-1} hr^{-1})	0.83	33.50	14.13	12.80	8.19	58	0.193	-0.885
DOC (mg kg^{-1})	56	634	266	207	171	64	0.809	-0.469
Biomass N (mg kg^{-1})	128	997	414	293	244	59	0.924	-0.378
DON(mg kg^{-1})	31	179	83	70	44	53	0.712	-0.761
DIN (mg kg^{-1})	1	23	10	11	5	50	-0.005	-0.434
N mineralization (mg kg^{-1} day^{-1})	0.9	6.7	2.6	2.2	1.6	62	0.894	-0.101
qCO_2	0.08	12.32	2.63	2.12	2.20	84	2.333	6.868
C/N biomass	0.56	15.93	3.60	3.03	2.24	62	2.452	9.077
Soil humidity	33	840	343	310	191	56	0.505	-0.265
Sphagnum (g m^{-2})	0	138	17	0	33	194	2.104	3.573
Racomitrium (g m^{-2})	0	131	28	19	34	121	1.266	0.892
Lycopodium (g m^{-2})	0	93	7	0	19	271	2.809	7.415
Deschampsia (g m^{-2})	0	91	20	18	17	85	1.645	3.796
Agrostis (g m^{-2})	0	199	59	50	46	78	0.587	-0.457
Eriophorum (g m^{-2})	0	14	1	0	3	300	2.166	4.095
Vaccinium (g m^{-2})	0	107	17	12	20	118	1.805	4.182
Carex (g m^{-2})	0	130	5	0	14	280	7.017	58.392
Total plant biomass (g m^{-2})	129	176	153	155	13	8	-0.058	-0.9112

Table 2. Model type and parameters (C_0 = nugget value; C_1 = sill) for indicated semivariograms.

Parameter	Model	C_0	C_1	$C_0/(C_0+C_1)$	range (cm)	r^2
Biomass C	Spherical	294440.0	294549.0	0.50	690.3	0.775
Respiration			Pure nugget effect			
DOC	Spherical	6925.1	19195.5	0.27	800.0	0.887
Biomass N	Spherical	49498.9	9738.0	0.84	600.0	0.074
DON	Spherical	601.5	1161.4	0.34	800.0	0.742
DIN			Pure nugget effect			
N mineralization			Pure nugget effect			
qCO_2	Spherical	2.5	5.5	0.31	319.8	0.436
C/N biomass	Spherical	4.2	1.4	0.75	357.4	0.028
Humidity	Spherical	20138.9	15131.3	0.57	600.0	0.449
Sphagnum	Exponential	86.1	1141.5	0.07	600.0	0.794
Racomitrium	Spherical	400.0	694.5	0.37	327.3	0.158
Lycopodium	Spherical	153.5	265.8	0.37	536.9	0.643
Deschampsia	Spherical	59.8	209.0	0.22	282.4	0.040
Agrostis	Spherical	1190.9	771.1	0.61	600.0	0.395
Eriophorum			Pure nugget effect			
Vaccinium	Spherical	184.5	164.9	0.53	404.3	0.258
Carex	Spherical	84.9	100.3	0.46	600.0	0.298
Total plant biomass	Spherical	0	152.3	0	356.1	0.537

The most formal way to analyse spatial variation statistically is by means of semivariance analysis and here it revealed the presence of spatial structure in variance for many of the parameters measured. Generally, the spatial dependence found was quite low (see Table 2). There was no evidence for spatial structure in soil respiration, DIN and N mineralization at the 2 m sampling interval. The remaining soil properties showed very week spatial dependence, with the exception of DON, DOC, biomass-C and *Sphagnum*. In the case of vegetation, Sphagnum showed the most important spatial correlation. Therefore, the pattern of spatial dependence of the studied soil properties and plant biomass was described by a nugget component, i.e. no spatial dependence or by spherical or exponential models plus a nugget component (Table 2). The fit of the

models to experimental semivariograms was rather poor, except for DON, DOC, biomass-C and *Sphagnum*, again. *Sphagnum* abundance showed a statistically significant negative correlation with both, DON and DOC. The ranges of spatial dependence found for DOC, DON, biomass C and *Sphagnum* varied from about 320 to 800 cm meaning that for distances longer than these the data were not spatially correlated. Samples taken 8 m apart will not exhibit errors due to spatial dependence. Selected examples of spatial distribution are mapped in Figure 1. Kriging maps for DOC and for *Agrostis* illustrate a patchy structure, where the average diameter of the patches is represented by the range of spatial dependence. In contrast, the map for respiration shows no detectable pattern of variation as individual high and low values may occur as neighbours which lead to a pure nugget effect model of spatial dependence. Mapping of the soil properties and vegetation is useful in the description and interpretation of interaction between them.

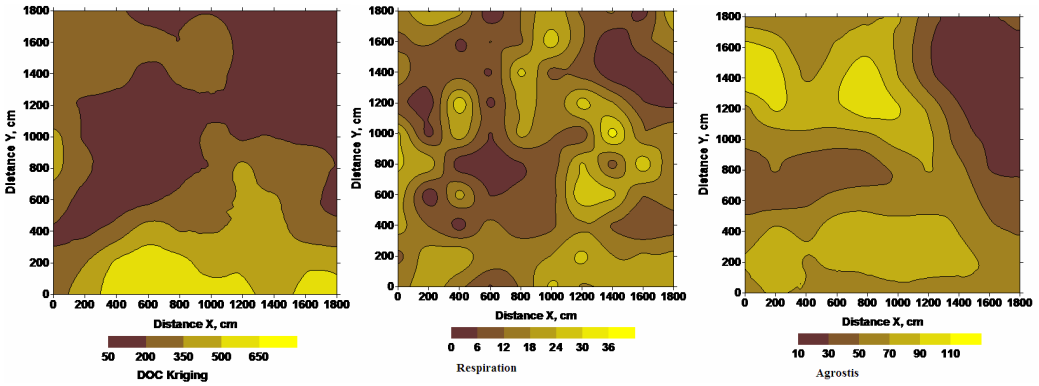

Figure 1. Maps for DOC, respiration and *Agrostis*.

Conclusion

Our study has showed the high levels of spatial complexity in soil properties and vegetation patterns that prevail in unimproved upland grasslands, and suggests that a complex set of interactions influence the spatial patterns of different soil microbial properties. Soil biological properties showed a wide difference in values at the small plot scale and high coefficients of variation. Individual plant species biomass was strongly skewed (asymmetric), whereas total plant biomass was rather homogeneous. In general the variograms of the soil properties and plant biomass were flat or nearly flat, and therefore spatial structure was absent or scarce (exceptions were DON, DOC, C-biomass and *Sphanum*). Our study suggests that a better understanding of spatial patterns of soil microbial properties requires careful study of the vegetation cover around the samples.

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Unravelling upbuilding pedogenesis in tephra and loess sequences in New Zealand using tephrochronology

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Abstract
The genesis of soils developed in either tephra or loess on stable sites differs markedly from that of soils developed on rock because classical topdown processes operate in conjuction with geological processes whereby material is added to the land surface so that the soils form by upbuilding pedogenesis. Understanding the genesis of such soils (typically Andisols and Alfisols, respectively) often requires a stratigraphic approach combined with an appreciation of buried soil horizons and polygenesis. In New Zealand, calendrically-dated tephras provide an advantage for assessing rates of upbuilding through chronostratigraphy. Many Andisol profiles form by upbuilding pedogenesis as younger tephra materials are deposited on top of older ones. The resultant profile character reflects interplay between the rate at which tephras are added to the land surface and topdown processes that produce andic materials and horizons. In loess terrains, upbuilding pedogenesis since c. 25,000 years ago is associated with maximum rates of loess accumulation c. 3─10 mm per century, sufficiently slow for soil-forming processes to continue to operate as the land surface gradually rises. Thus, Alfisol subsoil features are only weakly developed and Bw or B(x) horizons typically are formed. In contrast, topdown pedogenesis is associated with minimal or zero loess accumulation, the land surface elevation remains essentially constant, and subsoil features become more strongly developed and Bg, Bt, or Bx horizons typically are formed.

Key Words
Soil-sediment, tephrochronology, chronostratigraphy, pedostratigraphy

Introduction
Most text books describe pedogenesis in terms of classical 'topdown' processes that progressively modify a stable, pre-existing parent material. Indeed, modelling of such processes in the context of explaining soil development in time and space is almost invariably restricted to soils formed on rock, that factor being 'constant' apart from change resulting from in situ weathering (e.g., Minasny *et al.* 2008). However, in many landscapes, such as those of alluvial plains or where tephras or loess have been deposited, aggrading parent materials are very common. The evolution of soils in such landscapes therefore has an additional complexity because the impact from topdown processes is modified by the rates at which new materials are added to the landsurface via geological processes. The resultant soils are formed by upbuilding pedogenesis instead of topdown pedogenesis (Johnson *et al.* 1987; Almond and Tonkin 1999; Schaetzl and Anderson 2005). They may show distinctive layering and buried horizons, forming multisequal profiles. In this paper we use tephrochronology to examine the rates and processes involved in the evolution of late Quaternary soils via upbuilding pedogenesis from tephras (typically forming Andisols) and from loess (typically forming Alfisols) in New Zealand. Such application has been enhanced by the development of new calibrated age models for tephras erupted in the past c. 30,000 years (Lowe *et al.* 2008a).

Upbuilding pedogenesis on tephra
The accumulation at a particular site of numerous tephra deposits from sequential eruptions from one or more volcanoes leads usually to the formation of Andisols with distinctive layered profiles and buried soil horizons. Such layered profiles, together with their andic soil properties and glass content, are key features of Andisols. Study of the layers and attaining ages for them (tephrostratigraphy) is an important aspect of understanding Andisol formation. During periods of quiescence between major eruptions, soil formation takes place, transforming the unmodified tephra materials via normal topdown pedogenesis in a downward-moving front to form subsoil horizons. However, when new tephras are added to the land surface, upbuilding pedogenesis takes place. The frequency and thickness of tephra accumulation (and other factors) determine how much impact the topdown processes have on the ensuing profile character, and if 'developmental' or 'retardant' upbuilding, or both, will take place. Two contrasting scenarios can be considered.
In *scenario 1*, successive thin tephra deposits (ranging from millimetres to centimetres in thickness)

accumulate incrementally and relatively infrequently so that developmental upbuilding ensues. Such a situation occurs typically at distal sites. The thin materials deposited from each eruption become incorporated into the existing profile. Topdown pedogenesis continues as the tephras accumulate but its impacts are lessened because any one position in the sequence is not exposed to pedogenesis for long before it becomes buried too deeply for these processes to be effective as the land surface gently rises (Figure 1). This history thus leaves the tephra materials with a soil fabric inherited from when the tephra was part of the surface A horizon or subsurface Bw horizon (Lowe and Palmer 2005; McDaniel *et al.* in press). Each part of the profile has been an A horizon at one point, as illustrated in Figure 1.

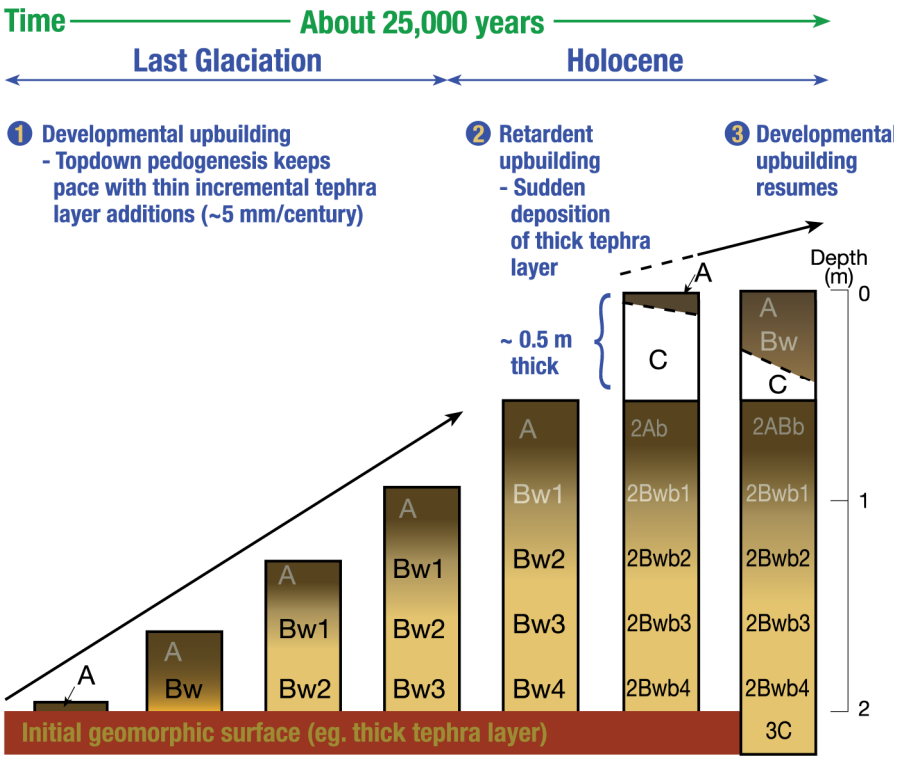

Figure 1. Model of upbuilding pedogenesis in tephra deposits and the formation of a multisequal Andisol over c. 25,000 years. In phase 1 (developmental upbuilding), thin, distal tephras accumulate slowly whilst topdown processes imprint weak horizonation features on them as the land surface gradually rises. In phase 2 (retardant upbuilding), the sudden deposition of a tephra layer ~0.5 m thick from a particularly powerful eruption buries the antecedent soil , isolating it from most surface processes so that topdown processes begin anew on the freshly deposited tephra. In phase 3, incremental tephra deposition on the new soil continues and developmental upbuilding resumes (after McDaniel *et al.* in press).

In *scenario 2*, tephra accumulation is more rapid, as occurs in locations close to volcanoes or when a much thicker layer (more than a few tens of centimetres) is deposited from a powerful eruption. In the latter case, the antecedent soil is suddenly buried and isolated beyond the range of most soil-forming processes (i.e., it becomes a buried soil/buried soil horizons). A new soil will thus begin forming at the land surface in the freshly deposited material. This scenario typifies retardant upbuilding, which means that the development of the now-buried soil has been retarded or stopped, and the pedogenic 'clock' reset to time zero for weathering and soil formation to start afresh. An example of a multisequal Andisol profile formed via retardant upbuilding pedogenesis since c. 9500 years ago is shown in Figure 2. Each of five successive tephra deposits (named Rotoma, Whakatane, Taupo, Kaharoa, and Tarawera) shows the imprint of topdown pedogenesis, as depicted by their soil horizonation. But the sudden arrival of each new deposit buries and effectively isolates each of the weakly-developed 'mini' soil profiles as the land surface rises. The soil in Figure 2 (Rotomahana series) is an Udivitrand in North Island, New Zealand. Retardant and developmental upbuilding may both occur in the evolution of a single Andisol profile. For example, in Figure 1, topdown pedogenesis effectively keeps pace with incremental tephra additions (at c. 5 mm per century) until interrupted by deposition of a thick layer that overwhelms the pre-existing soil, leaving an abrupt, clear boundary.

Upbuilding pedogenesis in loess

As recognised c. 120 years ago by James Hardcastle in the South Island of New Zealand, loess deposits commonly comprise multiple sheets with buried soils, formed during phases of very slow or zero loess deposition, marking the boundaries between sheets. In some areas, the loess-buried soil horizon sequences have been considered to represent cold-warm climates, respectively, with the change from one to the other analogous to an on/off switch.

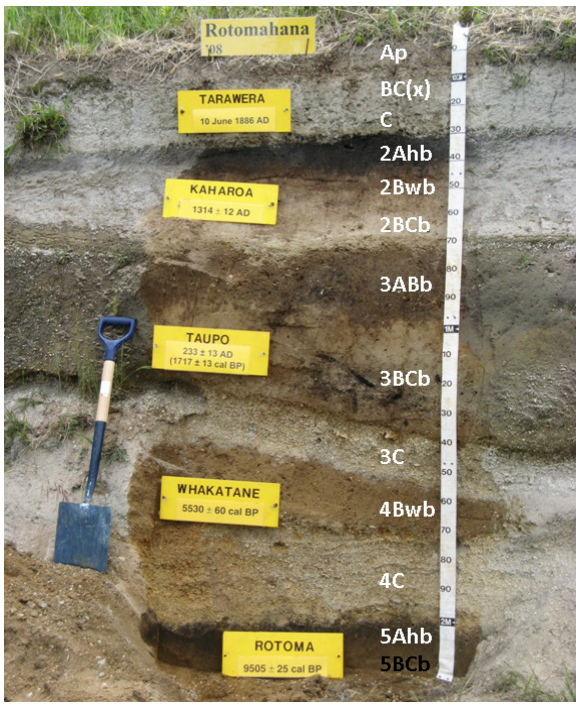

Figure 2. Example of a multi-layered Andisol formed through retardant upbuilding in New Zealand. After each tephra is deposited, soil begins to develop until it is buried by another tephra and topdown pedogenesis begins operating in the fresh deposit. The buried 'mini' soil profile on Whakatane tephra (4Bwb, 4C) reflects ~3800 years of pedogenesis, the amount of time it was at the land surface before burial by Taupo tephra; that on Taupo tephra (3ABb, 3BCb, 3C) reflects ~1100 years; that on Kaharoa tephra (2Ahb, 2Bwb, 2BCb) reflects ~570 years; and the topmost (surface) mini profile (Ap, BC(x), C) reflects ~125 years of pedogenesis on hydrothermally altered, mud-rich tephra deposited in AD 1886 by the Tarawera eruption. Some properties of the buried soil horizons may have been altered via diagenesis. The black 2Ahb horizon reflects a high content of type-A humic acids and charcoal following invasion by bracken fern and grasses after Polynesian deforestation and probably ongoing burning (after McDaniel *et al.* in press).

This model applied to the southern North Island area where cold climatic conditions (e.g., oxygen isotope stages [OIS)] 2, 6, 8) corresponded to maximum loess accumulation and relatively slow pedogenesis, and warm climatic conditions (e.g., OIS 1, 5, 7) to relatively fast pedogenesis and no loess accumulation (Palmer and Pillans 1996). Where loess accumulation is minimal or nil, soil development operates as a classical topdown process to form the distinctive subsoil (i.e. B horizon) features used to identify buried soils and to subdivide the loess column into sheets or soil stratigraphic units. But, as for distal tephra fallout sequences, most loess deposits have features indicative of continual pedogenesis. During periods when loess is accumulating, soil formation does not stop, but its effects are lessened as it eventually becomes buried too deeply for these topdown processes to be effective (Lowe *et al.* 2008b). This upbuilding history leaves the loess deposit with a soil 'vermiform' fabric inherited from when the loess was at the land surface and represented by soil A horizons. These vermiform features include fragipans, the interiors of which have a soil fabric throughout comprising traces of faunal activity such as back-filled burrows and root traces. The latter are very obvious where secondary $CaCO_3$ in the loess has formed root pseudomorphs. (The vermiform fabric is one of the signatures used to distinguish loess from other silty sediments such as weathered siltstones.) Soil formation thus occurs simultaneously with slow loess accumulation, forming a 'soil-sediment' via upbuilding pedogenesis (Figure 3). In New Zealand, the average rates of net loess accumulation since deposition early in OIS 2 of the widespread marker bed the Kawakawa tephra c. 27,100 years ago, and before the Holocene, are only about 3 to 10 mm per century (Eden and Hammond 2003; Lowe *et al.* 2008a, 2008b). When loess accumulation slows further or ceases altogether, topdown soil formation takes over. The imprint of topdown pedogenesis is more marked in the long run, forming the distinctive buried soil features – not simply because of 'improved' climatic conditions but because the rate of loess accumulation is so reduced that pedogenic processes and weathering effectively operate for longer periods. This model of alternate upbuilding pedogenesis and topdown pedogenesis phases applies widely to loess sequences in the South Island and probably in most of southern North Island (Lowe *et al.* 2008b).

In landscapes upwind from the main tephra sources in central North Island, the intermittent fallout of thin, distal tephra deposits at about 1 to 5 mm per century is at a rate comparable to slow loess accretion during glacial periods. Hence, for almost all of the time, upbuilding pedogenesis predominates in many distal-tephra-derived Andisol profiles because the accretion of tephra – together with tephric loess during glacials – is effectively continual. Typically, a few millimetres or centimetres of ash are deposited every few hundred years on the average, more frequently if cryptotephras (glass-shard concentrations not visible as layers in the field) are considered. The topdown-dominant phase only comes into play when a thicker tephra layer (approximately 20–30 cm or more) is emplaced so that the antecedent soil is effectively buried and sealed off. But in time, upbuilding pedogenesis will gradually resume as the ongoing eruptions of wind-borne (hence loess-like) tephras continue to 'dust' the imperceptibly rising land surface over thousands of years.

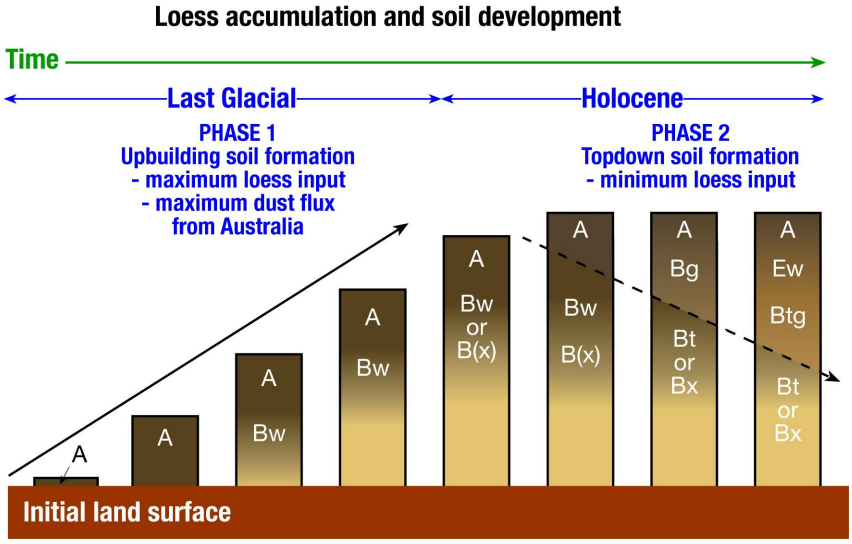

Figure 3. Model of soil development in loess since c. 25,000 years ago. The inital geomorphic surface approximates the Kawakawa tephra. Phase 1 depicts upbuilding pedogenesis during maximum (but slow) loess accretion (OIS 2); phase 2 depicts topdown pedogenesis with minimal or zero loess accretion (OIS 1). Soil horizons show that the maximum development of subsurface features occurs in phase 2 with more strongly developed horizons evident (after Lowe *et al.* 2008b).

Conclusion

Andisol profiles commonly have distinctive layering and buried soil horizons and form by upbuilding pedogenesis as younger tephra materials are deposited on top of older ones. The resultant profile character is determined by the interplay between the rate at which tephras are added to the land surface and topdown processes that produce andic materials and horizons. Understanding Andisol genesis thus often requires a stratigraphic approach combined with an appreciation of buried soil horizons and polygenesis. In loess terrains, upbuilding pedogenesis is associated with maximum rates of loess accumulation (during cold climates) but these rates are sufficiently slow for soil-forming processes to continue to operate as the land surface gradually rises 'millimetre by millimetre'. Thus, subsoil features are only weakly developed and Bw or B(x) horizons are formed. In contrast, topdown pedogenesis is associated with minimal or zero loess accumulation (during warm climates), the land surface elevation remains essentially constant, and subsoil features become more strongly developed so that Bg, Bt, or Bx horizons are formed. Loess accumulation and soil formation may be envisaged as 'competing' processes (e.g., see Muhs *et al.* 2004), but the former seldom exceeds the latter. Quantitative modelling of soil development should incorporate soils developed via upbuilding pedogenesis as well as those that evolve through topdown pedogenesis.

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Visualising the effect of compaction on root architecture in soil using X-ray Computed Tomography

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Abstract
The effect of soil compaction on the root architecture of the genetic model plant, *Arabidopsis thaliana*, was investigated using novel techniques which allow visualisation and quantification of undisturbed root architecture over time, and elucidation of responses to specific soil-based stimuli. X-ray Computed Tomography (CT) is an exciting tool for the study of root-soil interactions and offers major benefits. These include non-destructive measurements and 3-D visualisation, which are vital to advance our understanding of the dynamic nature of soil-plant interactions. Two soil types, a Newport series light sandy brown soil and a Worcester series heavy clay, were uniformly compacted to provide bulk densities of 1.1, 1.4 and 1.6 g/cm^3. Soil columns were scanned using a Nanotom® X-ray micro CT scanner at a resolution of 17 µm to enable the fine roots of *A. thaliana* to be visualised. The images were analysed and root structures were quantified. Destructive analysis of root architecture was undertaken for comparison with the X-ray CT images. Root architecture differed between treatments, and compaction had a marked effect on total root mass and root architecture. Percentage porosity and average pore size decreased as compaction increased.

Introduction
Soil compaction creates unfavourable conditions for root growth by limiting water, nutrient and oxygen supplies and increasing mechanical impedance (Cook *et al*. 1996). Lipiec *et al*. (1991) showed that roots became increasingly restricted to the upper soil horizons and decreased in length as bulk density increased. Root architecture is an important consideration as it determines the ability of root systems to acquire water and nutrients (Lynch 1995) and so influences overall productivity. As soil is a heterogeneous medium, effects on root architecture may influence the ability of plants to locate and absorb water and nutrients. Effective use of water and nutrients by plants is of vital importance as the availability of these resources may become increasingly scarce in future years.

As described by Whalley *et al.* (2000), there is an urgent need for non-invasive techniques capable of analysing the physical interactions between roots and the surrounding soil. Our understanding of how roots interact with their immediate soil environment largely remains a mystery as the opaque nature of soil has previously precluded *in situ* visualisation of undisturbed roots (Perret *et al*. 2007). New developments in non-invasive techniques such as X-ray CT provide an exciting opportunity to examine detailed root architecture in three dimensions for the first time (Tracy *et al*. 2010).

X-ray CT overcomes some of the limitations associated with previous methodologies for studying roots by providing non-invasive 3-D images (Heeraman *et al*. 1997; Pierret *et al*. 2002; Lontoc-Roy *et al*. 2006). The ability to view intact soil cores in this way enables accurate non-destructive quantification of soil parameters such as pore connectivity and tortuosity (Mooney 2002). Previous commonly employed destructive methods such as root washing cannot provide detailed information on root architecture, including branching characteristics and extension rate, which are inherently linked to conditions within the soil matrix.

Previous studies of roots using X-ray CT have often used substrates containing little organic matter (e.g. homogeneous sand or loamy sand) as growth media because the attenuation coefficient for root material is typically similar to that of other soil organic matter, making it difficult to visualise roots in the images obtained. However, as stressed by Gregory and Hinsinger (1999), our current need is for research involving complex growth media such as soil, as opposed to hydroponics, gels and sand-culture, to represent field conditions more closely. For this reason the experiment described here involved the use of field soils. *A. thaliana* was chosen because it is the genetic model plant, yet few experiments have investigated its root architecture when growing in heterogeneous substrates such as field soils.

Wild type *A. thaliana* plants and mutant lines in which root architecture was altered relative to the wild type were used. The soil was scanned at several growth stages to visualise temporal changes in root architecture and responses to a specific soil-based stimuli.

Methods

Plants were grown in columns of soil obtained from the University of Nottingham experimental farm at Bunny, Nottinghamshire, UK (52.52 ° N, 1.07 ° W). The two soil types used were a Newport series light sand (brown soil) and a Worcester series heavy clay (argillic pelosol). The soils were sieved to <2 mm before being uniformly compacted to provide bulk densities of 1.1, 1.4 and 1.6 g/cm^3. The soil columns were scanned using a Nanotom® X-ray micro computed tomography (CT) scanner at 100 kV, 210 µA to give a resolution of 17 µm. This high resolution permitted fine roots of *A. thaliana* to be identified. The image slices obtained were reconstructed to provide a 3-D visualisation of the soil column. The resultant images were then analysed to quantify soil physical parameters, including porosity, mean pore size and number of pores. Root length, tortuosity of the root path and root angle were also determined. Tortuosity of the root path can only be calculated using 3-D visualisation techniques as traditional destructive methods do not allow such information to be obtained. Manual root extraction methods were undertaken using Avizo® and VG Studio MAX®. The manual extraction methods involved selecting a specific range of grey pixel values believed to represent root material and isolating these from the rest of the sample. Destructive analysis of root architecture was undertaken using WinRHIZO® software for comparison with the X-ray CT images. Morphological characteristics including root length, diameter, area and volume were determined automatically. The results were analysed by two-way general analysis of variance (ANOVA) using Genstat 12.1.

Results

Root architecture differed between treatments and bulk density had major effects on total root mass and the 3-D architecture of the root systems. As the severity of compaction increased, percentage porosity and mean pore size decreased. Root lengths were measured using the polyline tool in VG Studio MAX® software (Figure 1). This tool can trace individual roots by following the shape of the root path, thereby providing accurate measurements of the length of roots as they extend down the soil column and encounter obstacles which may impede growth. Such measurements, made whilst roots are still encased within the soil matrix, are only possible using non-destructive approaches. Soil columns were also scanned before roots were present to visualise the original soil structure (Figure 2) and allow changes resulting from root growth to be identified. This approach enabled specific obstacles to root growth and the manner in which individual roots overcame these to be observed. For example, in Figure 3 an individual A. *thaliana* root can be seen bending round a 3.21 mm diameter mineral grain. This level of detail cannot be achieved using traditional methods for root studies.

Conclusions

In summary, X-ray CT is an exciting tool for the study of root-soil interactions which offers major benefits, and is vital to the advancement of our understanding of the dynamic nature of soil-plant interactions. X-ray CT studies provide further insights into the nature and limitations of root growth in different soil types and under contrasting edaphic conditions. The technology reported here has considerable potential to enhance our understanding of how the immediate soil environment affects root architecture. The present study has shown that compaction has a significant impact on root growth and further experiments will be undertaken to increase our knowledge of how root growth may influence soil structure over time. By exploiting innovative techniques such as X-ray CT, the below-ground impact of genotype on phenotype and, in particular, interactions between roots and their soil environment can be visualised. Such research may pave the way towards identification of novel genes with an important role in optimising the acquisition of water and nutrients to drive future crop breeding initiatives and enhance food security.

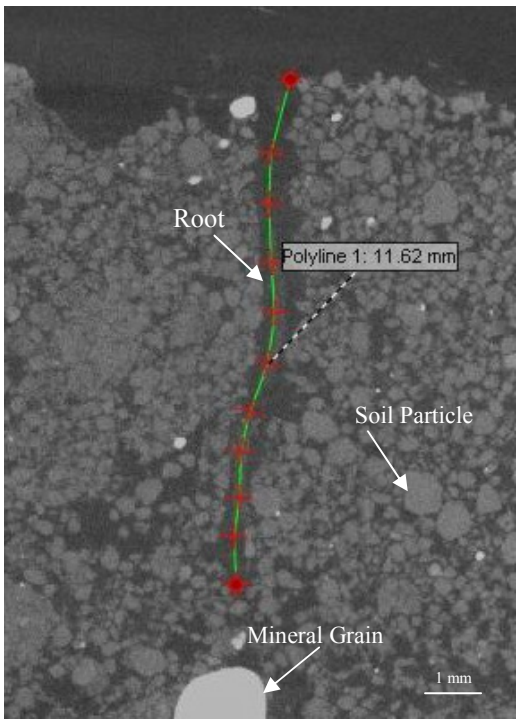

Figure 1. The root path of an individual *A. thaliana* root was traced through a column of Newport series loamy sand using the polyline tool in VG Studio MAX® software. Root length was 11.62 mm. Measuring the straight linear distance (10.08 mm) of the root provided a tortuosity value for the root path of 1.15.

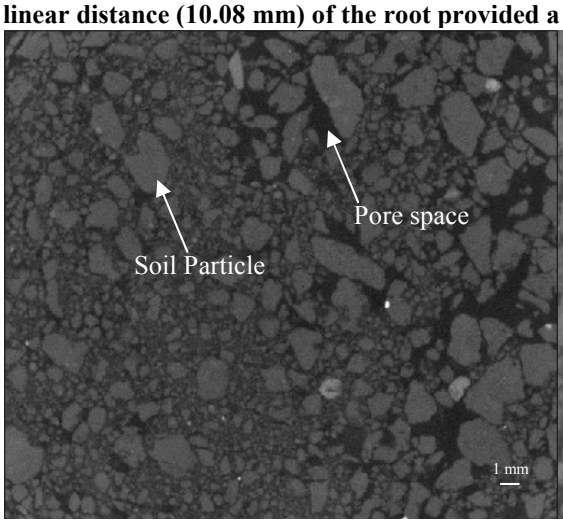

Figure 2. Image slice of a clay loam at 3-5 % moisture content. Porosity = 44.6 %, total pore count = 2183, total pore area = 107.25 mm^2, mean pore area = 0.049 mm^2.

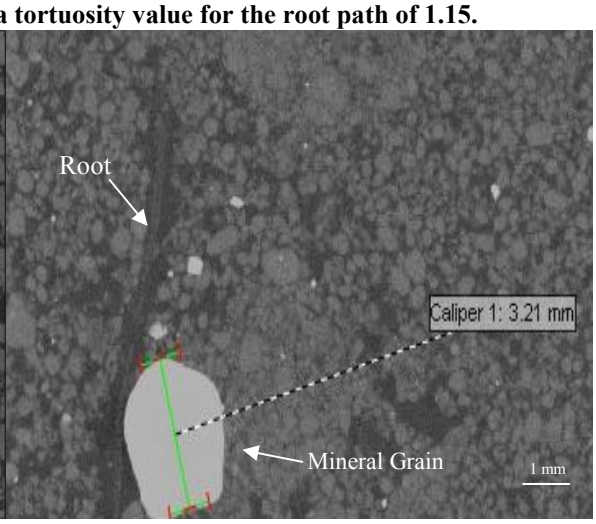

Figure 3. X-ray CT image of a root growing in a Newport series loamy sand. The root can be clearly seen growing around a 3.21 mm diameter mineral grain, measured using the caliper tool in VG Studio MAX® software.

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Wheat Root length density as affected by nitrogen treatment

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Abstract
Rooting density in the subsoil is one of the important factors in determining nutrient uptake by plants. In order to investigate the effect of different nitrogen treatment on root length 5 nitrogen and two water treatments were carried out with 3 replicates of each treatment. The experiment was designed as a completely randomized block. The root length and root dry weight were measured during the growing season at different depths in the soil. The results show an increase in the root length in the subsoil (60-80 cm depth) and a decrease in root length in the topsoil after anthesis. An important issue, therefore, is whether the uptake of NO_3^--N from subsoil will be limited by root length density.

Key Words
Wheat, root, length, density, nitrogen.

Introduction
A better knowledge of the distribution of roots in soil is necessary to ascertain their effect on water and nutrient uptake by plants. In agriculture, it is usually considered an advantage for crop plants to have deep roots. In many cases, the supply of water under drought conditions may be safeguarded by the uptake of water from the subsoil. But the question also arises as to whether the roots in the deeper layers contribute to the mineral nutrition of the crop to any worthwhile extent. The majority of roots are in top 60-cm layer but in semiarid climates often all the water in this zone is used up by grain filling time and it is roots in the lower layer that facilitate the filling of heads (Hurd and Spratt 1975). In field experiments in South Australia, Schultz (1974) found an average of nearly 50% of the roots in the 0-15 cm layer. The surface soil is normally dry after September in South Australia when wheat is at the heading stage (Large 1954), and the ability of plants to extract water from the subsoil is important for final yield. To fully extract the water from subsoil, Wind (1961) calculated, from the theory of unsaturated water flow, which root density has to be approximately 1 to 2 cm/cm^3. Roots near the surface are prone to desiccate and die; this would retard the ability of these rooting systems to make efficient user of rain from summer thunder storms (Russell 1973).Roots in the subsoil have potential value in feeding the plant, provided plant nutrients are available. Their contribution to plant nutrition will further depend on the fertility of the subsoil layers, the water content of the layers and the amount of roots that have been able to develop in these regions. To explain the relationship between root length density and NO_3-N utilization further experimental efforts concerning different density of roots in the soil profile are necessary.

Methods
The experiment had a randomised complete block design with 3 replications. Factors tested were 5 N treatments, 3 harvest dates and two water regimes arranged in factorial combination. Wheat (cv. Molineux) was used in this experiment. The N treatments were: (N_0) no N; (N_1) 150 mg per pot as KNO_3 placed in the topsoil at sowing; (N_2) 75 mg placed in the topsoil and 75 mg in the subsoil at sowing; (N_3) 150 mg placed in the subsoil at sowing and; (N_4) 75 mg placed in the topsoil at sowing and 75 mg placed in the subsoil on week after anthesis. The topsoil applications were applied to the surface of the soil which was then lightly cultivated. The plants were harvested at tillering, anthesis and maturity. The plants that did not receive N (N0) were harvested only at maturity. The two water treatments, introduced when the wheat was at anthesis, were surface irrigation sufficient to keep water stress low in the plants (W_1), and no surface water, but subsoil irrigation at 60 cm depth (W_2). The soil from each pot was separated into depths of 0-10, 10-20, 20-40, 40-60, 60-80 and 80-100 cm. A sub sample of soil was taken from each layer to determine water content and NO_3-N concentration by the method of Best (1976). Roots were separated from the remaining soil sample by flotation and root lengths and root dry weight were measured. The total length of root in each sample was estimated by the line intercept method of Tenant (1975).

Results
Roots grew slowly until tillering, then root length density increased sharply from tillering to anthesis,

especially in the surface layers. At maturity the root length in the topsoil decreased while it increased in the subsoil. This agrees with the observation by Campbell etal. (1977), who reported that there was a decrease in root length in the topsoil sometimes after anthesis and before the dough stage (Table 1). Neither the addition of N nor its placement affected root length density. The results presented by Lotfollahi (1996) imply that growth and distribution of roots in the subsoil have major roles in the post-anthesis N economy of plants because substantial uptake of NO_3-N did not occur until there was an increase in root length density.

Table 1. Root length density and root dry weight in the soil profile.[A]

Depth (cm)	Harvest		
	Tillering	Anthesis	Maturity
Root length density (cm/cm^3)			
0-10	7.10	14.49	9.46
10-20	4.50	12.43	8.55
20-40	3.37	6.85	6.34
40-60	1.98	4.65	3.92
60-80	0.21	1.52	2.31
80-100	0.05	1.16	2.30
l.s.d. (P=0.05)		1.36	
Root dry weight (g)			
0-10	0.63	1.75	1.38
10-20	0.36	0.92	0.88
20-40	0.51	1.41	1.20
40-60	0.39	0.99	0.89
60-80	0.08	0.42	0.57
80-100	0.01	0.30	0.67
l.s.d. (P=0.05)		0.15	

[A] Values have been averaged over all N treatments because the effect of N was not significant.
l.s.d applies to the interaction between depth and harvest.

Root dry weight increased until anthesis, then decreased sharply in the top 10 cm of soil (Table 1). However root dry weight in the 60 to 100 cm depth increase up to maturity. The root dry weight of the plants treated with no N (N_0) or with 75 mg in the topsoil and 75 mg in the subsoil at sowing (N_2) under the subsoil irrigation regime was slightly lower at maturity compared with the plants under surface irrigation (Table 2).

Table 2. The effect of nitrogen and water treatments on toot dry weight (g) at maturity.

Water treatment	Nitrogen treatment				
	N_0[A]	N_1	N_2	N_3	N_4
Surface irrigation	0.87	0.93	1.07	0.83	0.91
Subsoil irrigation	0.67	0.95	0.87	0.93	02.95
l.s.d. (P= 0.05)			0.16		

[A] N_0= no N, N_1= 150 mg N topsoil at sowing, N_2= 75mg N topsoil and 75 mg N subsoil (60cm) at sowing, N_3= 150 mg N subsoil at sowing, N_4= 75 mg N topsoil sowing and 75 mg N subsoil after anthesis.

Conclusion

The root length density in the topsoil decreased at maturity compared with anthesis while it increased in the subsoil. Root in the subsoil has potential value in feeding the plant especially in the late season that the top soil is dry.

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