# Linkages between plant traits and soil ecology in the rhizosphere and through litter decomposition

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# Linkages between plant traits and soil ecology in the rhizosphere and through litter decomposition

Karst M. Brolsma

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## *Abstract*

A plant root arranges uptake of water and nutrients. However, a drawback of this connection of terrestrial plants with soil is that they cannot easily escape unfavourable conditions. Plants can interact with biota and affect processes to influence belowground conditions. For this thesis, I investigated plant-induced effects in the rhizosphere and through litter turnover on soil microbes, nematodes, and carbon and nitrogen cycling. Such knowledge can help us to assess potential unintended and unexpected effects of e.g. genetically modified plants. Overall, the findings confirm close and diverse associations of plants with belowground biota and related processes. In addition, the data revealed that it is difficult to control a potato cyst nematode (*Globodera pallida*) through biofumigation, which is the maceration and incorporation of Brassicaceae into soil. Furthermore, the findings revealed that a genetically modified potato did not had distinct belowground effects as compared to a selection of conventional potato varieties.

“Looking at the dark side, careful one must be...
For the dark side looks back.”

Yoda

Star Wars - Episode III
Revenge of the Sith (2005)

# Contents

# Chapter 1

## General Introduction

Karst M. Brolsma

## *1.1 Plant-soil linkages*

One important feature of terrestrial plant physiology is the anchoring in soil by plant's roots. This connection of vascular plants to soil does not only physically support the aboveground part, it also arranges the uptake of water and essential nutrients. A drawback of anchoring in soil is immobility. Mobility will increase once seeds have been produced and dispersed via various factors (Levin *et al*., 2003). However, the drawback of anchoring in soil might become a dilemma when a plant is challenged with unfavourable conditions. Plants can cope with adverse conditions via belowground interactions with biota and processes through litter decomposition and rhizodeposition. The extent of such belowground plant-induced effects are reflected by plant specific traits.

Plant traits have been defined as "morphological, anatomical, physiological, biochemical and phenological characteristics of plants and their organs. These characteristics determine how primary producers respond to environmental factors, affect other trophic levels, influence ecosystem processes and services and provide a link from species richness to ecosystem functional diversity" (Kattge *et al*., 2011, p.2907). Surprisingly, despite the entity of traitbanks like TRY (Kattge *et al*., 2011), a clear shift between belowground plant traits and aboveground plant traits is more than evident as the soil observations are under-represented (Mulder *et al*., 2013). However, in applied soil ecology these belowground plant traits are by far the most relevant.

The continuous search and selection of mankind for favourable plant traits has in fact laid the basis of agriculture. Desirable traits that improve yield, taste and resistance against pests and pathogens resulted in modern crop varieties. Modern crops also include genetically modified (GM) varieties and, depending on the nature of the modification, GM plants can have consequences for belowground ecosystems. Soil organisms and processes can be affected through litter quality and rhizodeposition that might differ between a modified and for example the unmodified parental variety. Insight in soil processes and biota in relation to (soil) plant traits is essential, for example in the risk assessment of a GM variety.

## 1.2 Soil Processes

### 1.2.1 Rhizodeposition

Rhizodeposition is the release of photosynthetically assimilated carbon (C) into the soil zone around a root (Jones *et al.*, 2004). This thin soil zone next to a root is called the rhizosphere and is under the influence of root growth through rhizodeposits (Rout, 2014). Via rhizodeposition a plant can release up to 27% of the C that is allocated to roots and approximately 11% of net fixed C by photosynthesis (Jones *et al.*, 2009). Rhizodeposits have several functions belowground, such as improving nutrient availabilities and influence plant growth-promoting bacteria and as such support plant growth.

Carbon flow in the rhizosphere is often assumed to be a single directional pathway. However C can also be taken up again by the roots, e.g. in the form of amino acids (Wilkinson *et al.*, 2014). Amino acids, together with organic acids and sugars, are the main root exudates of many plant species (e.g. Dakora and Phillips, 2002), and root exudates are important rhizodeposits. The quantity and composition of root exudates and rhizodeposits depends on biotic and abiotic stressors, but also on the age of individual plants and on plant traits (Cheng *et al.*, 2014 & Whipps, 1990).

Besides the general constituents, rhizodeposits in Brassicaceae comprise specific compounds such as glucosinolates. In the rhizosphere, glucosinolates are localized in the outer periderm layer of the root (McCully *et al.*, 2008 & Rumberger and Marschner, 2003). Glucosinolates and especially the degradation product isothiocyanates have belowground consequences upon biota (Bressan *et al.*, 2009 & Rumberger and Marschner, 2004 & Schreiner and Koide, 1992). The ecological role of glucosinolates is considered to be a defence system in close association with the enzyme myrosinase (Fahey *et al.*, 2001 & Halkier and Gershenzon, 2006). So glucosinolates have the potential to affect soil biota through rhizodeposition, and can therefore be used to quantify belowground effects of plant traits like glucosinolates on soil biota and related processes.

### 1.2.2 Litter decomposition

Litter decomposition is the biotic degradation and transformation of organic matter in soil and can be considered as a two stage process (Gessner *et al.*, 2010). Initially, litter is broken down into smaller pieces by detrivores, i.e. fragmentation, and subsequently these fragments are further reduced into organic and inorganic components by microorganisms. These components are taken

up by organisms or are removed from the soil system through leaching, runoff, or released in gaseous forms (Amundson, 2001). Drivers of decomposition are environmental characteristics, the composition of the decomposer community and the quality of the litter (Aerts, 1997 & Heal *et al.*, 1997).

Plants affect decomposition processes by the chemical and physical composition of its tissue. It was already observed in the 1920s that the C to nitrogen (N) ratio was a relevant index in decomposition (Waksman, 1924). Carbon and N that are tied up in organic matter through photosynthesis (C) and uptake and assimilation of nutrients (N), provide the energy and building blocks such as amino acids in protein synthesis. Similar as rhizodeposition, also litter quality is related to plant traits (Cornwell *et al.*, 2008 & Mulder *et al*., 2013). In agriculture, Brassicaceae are used in a process called biofumigation by means of incorporation of glucosinolate rich plant material in the soil (Morra and Kirkegaard, 2002). As such, inducing a peak of isothiocyanate release with the potential to control soil dwelling pests and pathogens (Kirkegaard and Sarwar, 1998).

### *1.2.3 Relevance of belowground processes*

Rhizodeposition and litter decomposition are important processes as 90% of terrestrial plant production ends up as organic matter (Cebrian, 1999). In addition, up to 50% of total $CO_2$ released from terrestrial ecosystems can be controlled by rhizosphere interactions between roots and soil biota (Cheng *et al.*, 2014). Rhizodeposition and litter turnover result in resource availability that interacts with belowground organisms and nutrient cycling. For example, microbial activity increases due to C and N input into the rhizosphere (e.g. Bulgarelli *et al.*, 2013). The microorganisms in the rhizosphere use the nutrients for their own metabolism and growth, respire $CO_2$ and affect nutrient availability. Eventually, part of the nutrients can be taken up again by plants and stimulate growth, performance and/or community composition of plants. This is also known as plant-soil feedback (Van der Putten *et al.*, 2013). Understanding belowground processes in relation to plant traits and biota will improve our ability to predict consequences of anthropogenically induced changes. For example, insight in soil processes and soil biota is key to assess possible (unintended and unexpected) risks of a genetically modified crop in agriculture (Bruinsma *et al.*, 2003). Next to that, glucosinolates have also been used to explore belowground pathways of invasive species in relation to soil biota (Cantor *et al.*, 2011 & Lankau *et al.*, 2011 & Lankau, 2012).

## 1.3 Microorganisms

A single gram of soil can contain up to $10^9$ individual microbial cells belonging to more than $10^6$ distinct taxa (Gans *et al.*, 2005 & Pietramellara *et al.*, 2002). Curtis and Sloan (2005, p.1331) stated that "exploring microbial diversity is becoming more like exploring outer space with soil representing a 'final frontier' that harbours a largely unknown microbial universe". Insight into the extreme diversity of microbial communities in soil is constrained by the aspect that cultivation is not possible for the majority (e.g. Hirsch *et al.*, 2010). Moreover, the small size and morphological similarity of microorganisms limits identification and classification (e.g. Nannipieri *et al.*, 2003). Over the past decades, molecular techniques have significantly increased our knowledge with respect to microbial diversity through characterization of cell components such as DNA and RNA (Bulgarelli *et al.*, 2013 & Mendes *et al.*, 2011 & Rappé and Giovannoni, 2003). However, activity and functional aspects of microbial communities were often not taken into account (e.g. Ellis *et al.* 2003). One of the challenges in soil ecology is to improve our understanding of soil ecosystem functioning, e.g. in relation to land use change.

### 1.3.1 Microbial activity

A hot spot of microbial activity in soil is the rhizosphere, due to rhizodeposits that enter the zone. Rhizodeposits do not only fuel microbial life, but can also selectively affect microorganisms (Sugiyama *et al.*, 2013). For example, microbial growth was differentially affected by species specific root exudates (Carvalhais *et al.*, 2013 & Zhang *et al.*, 2014). In addition to rhizodeposition, plant species specific litter qualities also affected microbial communities through litter turnover (Cornwell *et al.*, 2008). In general, organic compounds are used by microorganisms to yield energy for their metabolism and result in $CO_2$-fluxes and uptake of oxygen. Measurements of $CO_2$-fluxes (and oxygen) are often used as an index for microbial activities in soil ecosystems. Moreover, addition of C rich substrates (e.g. carbohydrates) and assessment of the microbial response gives insight into active microbial biomass, microbial catabolic capacity and diversity of soil ecosystems (Campbell *et al.*, 2003 & Degens and Harris, 1997).

### 1.3.2 Assessment of microbial catabolic diversity

In this thesis, we selected the MicroResp™ method (Campbell *et al.*, 2003) to assess catabolic activities of the microbial communities in relation to plant traits and soil processes. The

MicroResp™ system uses small soil volumes and various C rich substrates can be included to assess microbial responses making the system suitable for rhizosphere studies. Blagodatskaya and Kuzyakov (2013) described four microbial states of activity in soil ecosystems: active, potentially active, dormant and dead. The last state does not contribute to microbial activity, whereas all living microorganisms are included in the first three states. Microbes in the active state are involved in substrate utilization and associated transformations, such as nutrient cycling. The potentially active state can switch to the active states within minutes to a few hours. The dormant state does not contribute currently to these processes, but can switch due to altered circumstances over a longer period. The incubation period of the MicroResp™ was throughout this thesis no longer than 6 hours, and the measurements reflect the catabolic diversity of the active and the potentially active microbial communities.

## *1.4 Nematodes*

Another group of organisms reaching high densities and abundances in soil are nematodes. A single square meter of soil can contain millions of individuals, and a handful of soil can comprise up to 50 different species (Bongers and Ferris, 1999). Assessment of the composition of nematode communities has emerged as a useful tool to monitor environmental conditions related to soil ecosystem functioning (e.g. Ferris *et al*., 2001 & Neher, 2001). Nematodes have a diverse diet, ranging from bacteria, fungi, protozoa, algae, other nematodes and can even parasitize animals or plants (Yeates *et al.* 1993). Nematodes contribute to soil functions, for example through grazing of microorganisms and thereby stimulate nutrient turnover. It was observed that nematode species differentially affected bacterial community composition and as such increase bacterial biomass and C mineralization (Postma-Blaauw *et al.*, 2005). Nematode identification requires a considerable amount of experience and expertise and limits exploration of *in situ* free living nematodes. Recently, genomic research has made a considerable progress and resulted in the use of small subunit ribosomal DNA for nematode identification and quantification (Holterman *et al.*, 2006 & Vervoort, 2013).

Nematodes can also be a serious threat in agriculture. To illustrate this, severe infestation of potato cyst nematodes *Globodera* spp. can decrease potato yield up to 80% (Singh *et al.*, 2013). The cyst is a survival stage of the nematode, which is the body of a deceased female full with eggs. Within each egg, a second stage juvenile is present in diapause. Juveniles hatch from the cyst when stimulants are released through root exudates, such as solanoeclepin A in leachates of Solanaceae

(Schenk *et al.*, 1999). Control methods in agriculture for the potato cyst nematodes include breeding for resistant varieties, crop rotation and application of nematicides. In addition, there is potential to use biofumigation with Brassicaceae to control potato cyst nematodes (see Lord *et al*., 2011). A farmer can integrate crucifers in the crop rotation and incorporate it into the soil to suppress infestation of potato cyst nematodes. However, it is not yet known how effective such a control method is for *Globodera* spp. Most research on control of *Globodera* spp. by biofumigation did not include the survival stage of the nematode (cyst) and can as such limit the success of the method (Motisi *et al.*, 2010). Biofumigation using *B. juncea* was included to explore how effective incorporation of glucosinolate rich material controls *Globodera pallida*.

## *1.5 Objective and thesis layout*

Soil is fundamental and irreplaceable, and it supports biochemical cycling of nutrients and plant productivity in both natural and agricultural ecosystems (Nannipieri *et al.*, 2003 & Van der Heijden *et al.*, 2008). For this thesis, the main objective is to investigate plant-induced effects on soil biota and processes, and link the observed effects to specific, less investigated belowground plant traits. Exploring belowground plant traits, biota and processes can help us to understand and predict consequences of land use changes, for example the introduction of genetically modified crops. Moreover, such knowledge is also relevant to improve control methods of soil dwelling pests and pathogens in the case of biofumigation. To address the objective of this thesis, laboratory, greenhouse and field trials were conducted and findings of the trials are presented in the following chapters.

In Chapter 2, effects of plant species and plant genotype were assessed on soil microbial catabolic diversity in rhizosphere and in bulk soil. Plant species effects were investigated in a long term field experiment (>10 years). In addition, genotype effects were explored in agricultural trials including *B. juncea* and *Solanum tuberosum*, respectively.

Chapter 3 continues with the assessment of microbial catabolic diversities in the rhizosphere in relation to glucosinolate levels of *B. juncea* in a field experiment. Next to that, glucosinolate levels and microbial catabolic diversities were also linked with the nematode community composition in the rhizosphere by means of the molecular tool.

The aim of Chapter 4 was to investigate whether higher levels of glucosinolates could decrease the survival of encysted *G. pallida* by means of biofumigation, and explore factors that stimulate glucosinolate levels in *B. juncea* in a pot experiment. In addition, toxicity of allyl isothiocyanate was tested *in vitro* on encysted *G. pallida*, this isothiocyanate is the hydrolysed component of the main glucosinolate in *B. juncea*, i.e. sinigrin.

In Chapter 5, we assessed whether a GM potato affected belowground organisms and processes. The assessment was done by measuring the catabolic activities of soil microbes in rhizosphere and in bulk soil in a field experiment conducted with three *S. tuberosum* cultivars. Next to the field experiment, a decomposition experiment was conducted in the laboratory to investigate effects of decaying litters of the cultivars on C and N mineralization.

In Chapter 6, the observed effects of plant traits on soil biota and processes of the preceding chapters are summarized and discussed. The chapter is split in six sections. Section one is on plant-induced effects on rhizosphere microbial catabolic activities, the second section is on linkages between rhizosphere and bulk soil microbial catabolic diversities, the third section is on plant-induced effects on microbial activity during litter turnover, the fourth section is on the MicroResp™ methodology, the fifth section is on plant-induced effects on nematodes, and the chapter ends with concluding remarks

# Chapter 2

## Microbial catabolic diversity in and beyond the rhizosphere of plant species and plant genotypes

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## *Abstract*

The aim of this study is to assess plant species and plant genotype effects on soil microbial catabolic diversity in rhizosphere and in bulk soil. We studied grasses and herbs from an experimental grassland system and two crops, namely four *Brassica juncea* genotypes and five *Solanum tuberosum* genotypes grown in agricultural trials. The MicroResp™ system was used to explore plant induced effects on the microbial catabolic diversities. We selected substrates for the MicroResp™ based on root exudation profiles of related species and included 3 amino acids, 4 organic acids, 3 carbohydrates and a recalcitrant polysorbate in the system. Plant species and genotype specific effects were observed in the microbial catabolic response upon addition of carbon rich substrates. Plant species effects were not restricted to the rhizosphere, but were also reflected in bulk soil. Plant genotype effects were observed in the microbial response in the *S. tuberosum* rhizosphere, but such effects could not be detected for *B. juncea*. Our results show that the MicroResp™ system is sensitive to detect plant species and genotype-induced effects in the catabolic response of the microbial community upon addition of carbon rich substrates in rhizosphere and in bulk soil. Selection of substrates for the MicroResp™ that are related to plant root exudation profiles can help to improve our understanding further of belowground plant-induced effects on soil ecosystem functioning.

## 2.1 Introduction

Soil microorganisms fulfill major roles belowground, including nutrient cycling and decomposition of organic matter. Soil microbial communities in turn are affected by plants, soil and environmental factors (Degens *et al.*, 2000 & Fierer *et al.*, 2009 & Rout, 2014). Over the past decades, high throughput molecular techniques have significantly increased our knowledge with respect to microbial diversity through characterization of cell components such as DNA and RNA (Bulgarelli *et al.*, 2013 & Mendes *et al.*, 2011 & Rappé and Giovannoni, 2003). However, insight into the functioning of microbial communities in soil is constrained by the aspect that cultivation is not possible for the majority of species (Hirsch *et al.*, 2010). Still, assessment of the functional diversity via community level physiological profiling has been shown to improve our understanding of the microbial activities in soil ecosystems (Zak *et al.*, 1994 & Ellis *et al.* 2003 & Blagodatskaya and Kuzyakov, 2013).

The rhizosphere is a hot spot of soil microbial activity, which is due to carbon(C) rich rhizodeposits (Jones *et al.*, 2009). Rhizodeposition of C and a blend of other compounds occurs in all plant roots, but its quantity and quality depend on many factors, including plant species and plant genotype (Cheng *et al.*, 2013 & Gransee and Wittenmayer 2000 & Lesuffleur *et al.*, 2007). Plant rhizodeposits do not only fuel microbial life, but can also selectively affect microorganisms (e.g. Sugiyama *et al.*, 2013). For example, plant species specific root exudates differentially affected growth kinetics and motility of beneficial rhizosphere microbial strains (Carvalhais *et al.*, 2013 & Zhang *et al.*, 2014). Moreover, genotypes of *Solanum tuberosum* have been shown to exert distinct effects on microbial community structure in the rhizosphere (Weinert *et al.*, 2009). However, assessment of microbial diversity using molecular techniques does not reveal active functioning. Therefore, there is a need to investigate *in situ* activities of microbial communities. The role of microbial activities related to plant-induced effects is relevant in understanding soil ecosystem functioning. In addition, such knowledge can help to assess risks of anthropogenic changes, e.g. the introduction of genetically modified crops (Dini-Andreote and Van Elsas, 2013).

The aim of this study is to assess plant species and plant genotype effects on the microbial functional diversity in the rhizosphere and in bulk soil. We compared grassland species and genotypes of two agricultural crops: mustard and potato, including one genetically modified potato genotype. Microbial catabolic diversity is measured upon the addition of a variety of C rich substrates in the MicroResp™ system, resulting in a fingerprint of the microbial community functioning (Campbell *et al.*, 2003). Lalor *et al.* (2007) found that MicroResp™ was substantially better in separation of treatments as compared to the methodology on microbial catabolic diversity

developed by Degens and Harris (1997). The MicroResp™ system uses small volumes of soil, which makes the methodology suitable for rhizosphere studies. The measurements can be executed right after sampling and as such, are close to *in situ* observations. The following research questions are addressed to support the overall aim: (i) are there differences in microbial catabolic activities between rhizosphere and bulk soil as measured with the MicroResp™ system, (ii) is the microbial catabolic response in the rhizosphere affected by plant species and genotypes, and (iii) are potential plant induced effects different in rhizosphere and in bulk soil?

## 2.2 Materials and Methods

### Experimental design and soil sampling

We included three field experiments in which the catabolic diversity of the microbial communities in rhizosphere and bulk soil samples was assessed by the MicroResp™ system. The first one was a long-term experiment with monocultures of natural grassland species. Experiments 2 and 3 were agricultural ecosystems including mustard and potato genotypes, respectively (Table 2.1).

Rhizosphere sampling was similarly executed for all three experiments. Samples were taken by carefully lifting plants including root-adhering soil using a spade. Plants were shaken twice to remove non-rhizosphere soil and the still adhering soil, being rhizosphere soil, was subsequently brushed from the roots. Composite samples of bulk soil were taken using a soil corer (Ø2 cm upto 15 cm depth) in all experiments; roots and adhering soil were removed. We sampled 15 cores per plot in Experiment 1, 30 cores per plot in Experiment 2 and 16 cores per plot in Experiment 3.

*Table 2.1.*

Experimental layout of Experiment 1, 2 and 3 including plant species or genotypes and the selection of substrates used in the MicroResp™ system per experiment.

| Experiment | Species | Genotypes | Substrates |
|---|---|---|---|
| 1 | *Agrostis capillaris* | - | alanine, arginine, citric acid, fumaric acid, glucose, malic acid, oxalic acid, phenylalanine, sucrose, trehalose and tween-80 |
| | *Anthoxanthum odoratum* | - | |
| | *Centaurea jacea* | - | |
| | *Festuca rubra* | - | |
| | *Holcus lanatus* | - | |
| | *Leucanthemum vulgare* | - | |
| | *Plantago lanceolata* | - | |
| | *Rumex acetosa* | - | |
| 2 | *Brassica juncea* | Terrafit | glucose, malic acid, sucrose, trehalose and tween-80 |
| | | Terratop | |
| | | Terraplus | |
| | | ISCI 99 | |
| 3 | *Solanum tuberosum* | Aveka | glucose, malic acid, sucrose, trehalose and tween-80 |
| | | Aventra | |
| | | Désirée | |
| | | Karnico | |
| | | Modena | |

The number of cores differed among experiments due to differences in the experimental layout (see below). In Experiment 2, a relatively large number of cores were taken because bulk soil samples were also used for nematode community profiling (Vervoort *et al.*, 2014). All samples were refrigerated immediately after sampling (4°C). Measurement of the microbial catabolic responses was done within 24 h after sampling for each experiment.

## *Experiment 1*

Experiment 1 was conducted within a plant biodiversity experiment initiated in spring 2000 at an arable field in Wageningen, The Netherlands (Van Ruijven and Berendse 2003). The topsoil of the arable field was removed up to 50 cm depth, after which square wooden frames measuring 1 x 1 x 0.5 m (l x w x d) were inserted in the holes. The plots were filled with a mixture of a sandy arable field soil and pure sand (1/3 v/v) to adjust the nutrient status of the soil to the grasslands in which the study species typically occur. Organic matter content of the soil mixture was 1.3% and pH ($CaCl_2$) 7.3, averaged over all plots as measured in 2001. Plant species selected were four grasses (*Agrostis capillaris* L., *Anthoxanthum odoratum* L., *Festuca rubra* L., *Holcus lanatus* L.) and four herbs (*Centaurea jacea* L., *Leucanthemum vulgare* Lamarck, *Plantago lanceolata* L. and *Rumex acetosa* L.). A total of 144 plants per plot were planted as seedlings. The plant species composition was maintained by removing seedlings of all other species at monthly intervals during the growing season over a period of 11 years. We sampled the 48 monoculture plots of this experiment in March 2011, being eight different species distributed over six replications (blocks).

## *Experiment 2*

Experiment 2 comprised four *B. juncea* L. genotypes and was conducted in Münster, Germany. The soil was sandy with 1.3% organic matter and pH ($CaCl_2$) 6.4. Four *B. juncea* genotypes were selected (Terrafit, Terratop, Terraplus and ISCI 99) based on the varying concentration of the secondary metabolite 2-propenyl glucosinolate (sinigrin) in these genotypes (Vervoort *et al.*, 2014). After sinigrin hydrolysation toxic components, as such 2-propenyl (allyl) isothiocyanate, are produced which can detrimentally affect soil organisms (Olivier *et al.*, 1999). Seeds were sown in July 2010 in plots of 4 x 15 m using a complete randomized block design with four replications. Seed densities were 12 kg $ha^{-1}$ for genotype Terrafit, Terratop and Terraplus and 15 kg $ha^{-1}$ for ISCI 99. Plants were fertilized at sowing with 70 kg nitrogen $ha^{-1}$ and 17.5 kg sulphur $ha^{-1}$ and soil samples were taken in September 2010.

## *Experiment 3*

Experiment 3 comprised five potato (*S. tuberosum* L.) genotypes and was conducted in 2010 on an experimental field located in the province of Drenthe, The Netherlands. The soil was a sandy peat with 25.5% organic matter and pH ($H_2O$) 4.6. Potato genotypes Aveka, Aventra, Désirée, Karnico, and Modena, were planted in spring 2010. Modena is a genetically modified potato, with distinct starch characteristics which was bred from the parental genotype Karnico (De Vetten *et al.*, 2004). Seeds were sown in April and the field was fertilised with organic (pig manure) and inorganic fertilisers. In total the field received 179 kg N $ha^{-1}$, 81 kg phosphorus $ha^{-1}$ and 165 kg potassium $ha^{-1}$. The field consisted of four replicate plots per genotype, which were distributed using a randomized complete block design. Each plot contained 28 plants divided over four ridges. Soil sampling was done in September 2010.

## *Microbial catabolic diversity*

Assessment of the microbial catabolic diversity was done using the MicroResp™ procedure (Campbell *et al.*, 2003). Samples were not pre-incubated as would have been a normal procedure, since plant-induced rhizosphere effects might be lost during 48 h of pre-incubation due to their low mean residence times (<2 h) (Jones and Darrah, 1993). In all experiments we used the substrates glucose, sucrose, malic acid, trehalose and tween-80, and water as control. For Experiment 1, also alanine, arginine, citric acid, fumaric acid, oxalic acid and phenylalanine were included (Table 2.1). Substrate selection was based on root exudation profiles of agricultural plants and because some of the substrates have shown the capability to induce respiration across a range of soils (Table 2.2) (Campbell *et al.*, 1997 & Degens and Harris, 1997). Substrates were dissolved in demineralised water and prepared as stock solutions designed to deliver 30 mg C $g^{-1}$ soil solution after dispensing 25 µl of the solution. Based on the solvability, lower concentrations (7.5 mg C $g^{-1}$ soil solution) were used for substrates alanine, arginine, phenylalanine, oxalic acid and fumaric acid (Table 2.2). The substrate solution was dispensed into a deep-well plate, and each substrate was dispensed into four replicate wells.

Soil samples were sieved (2 mm mesh) to remove root parts and added to the deep-well using the standard filling device (Campbell *et al.*, 2003) to add 300 µl of soil to each well. Moisture content of the samples was determined (drying at 105°C for 24 h) and fresh soil weight added to the deep-well plates was measured to calculate the average weight of soil per well

Absorbance of the indicator dye was measured in the detection plate at 570 nm before and after the incubation period using a micro plate reader (VMAX, Molecular Devices). During incubation, the detection plate is connected (upside down) to the deep-well plate to measure respiration. Colour development (from pink to yellow) was normalized by subtracting the averaged time zero measurements for each plate from the measured colour development per well after 6 h incubation according to Campbell *et al.* (2003) and converted to $CO_2$ concentrations using a calibration curve; $\%CO_2 = 0.02 \cdot A_{570}^{-3.11}$, $R^2 = 0.93$. Where $\%CO_2$ (v/v) is the concentration in the headspace after incubation and $A_{570}$ is the normalized absorbance. Median $CO_2$ concentrations ($n = 4$) for each substrate per plot were converted to respiration rates (μg $CO_2$-C $g^{-1}$ soil $h^{-1}$) using soil fresh weight and soil water content. Median respiration rates of the control, i.e. water only, were subtracted from the median respiration rates induced by a C source.

*Table 2.2.*

Characteristics of substrates used in the MicroResp™ system, given the carbon (C) and nitrogen (N) atoms for each substrate and the effect of each pH in the stock solution after the substrate was dissolved.

| Substrate | Type | C-atoms | N-atoms | pH effect[a] |
|---|---|---|---|---|
| Alanine[b] | Amino acid | 3 | 1 | + |
| Arginine[b] | | 6 | 4 | ++ |
| Phenylalanine[b] | | 9 | 1 | + |
| Oxalic acid[b] | Organic acid | 2 | | - |
| Fumaric acid[b] | | 4 | | - |
| Malic acid | | 4 | | -- |
| Citric acid | | 6 | | -- |
| Glucose | Carbohydrate | 6 | | + |
| Sucrose | | 12 | | + |
| Trehalose | | 12 | | + |
| Tween-80 | Polysorbate | 64 | | + |

[a] change in pH of the stock solution after dissolving substrate over all experiments, + and – are changes in pH within 1.5 units, ++ and -- are changes in pH more than 1.5 units as compared to control (water)
[b] ¼ of the substrate amount was used due to solvability

## *Data analyses*

Average substrate-induced respiration (SIR) was calculated over all C sources for rhizosphere and bulk soil samples. The effects of plant (genotype or species) and soil (rhizosphere or bulk) on SIR were tested using ANOVA. Average SIR data of Experiment 1 was $^{10}$log-transformed to obtain a normal distribution and equal variances of the residuals. Least significant difference was used at the 5% level to identify differences between treatments in Experiment 1. Analysis of substrate-induced respiration was done using GenStat 16$^{th}$ edition.

MANOVA was used to identify effects of plant genotype and species on the microbial catabolic diversity in rhizosphere and bulk soil. When significant effects were observed in the MANOVA, discriminant analysis was conducted using the stepwise approach to identify dissimilarities and select the substrates that contributed most to the separation. Criteria, i.e. probabilities of the Wilks' lambda *F*-value, used in the stepwise approach of the discriminant analysis were entries of 0.1 and removals of 0.2. Discriminant scores of each function that separated plant groups were calculated and analysed by ANOVA followed by Tukey HSD to explore differences among plants further. Average SIR and standard errors were calculated for the substrates that contributed most (statistically significant) to the discriminant functions. For each of these separating substrates a ratio was calculated with the average SIR of the separated plant species (or genotype(s)) as numerator and the average SIR of the remaining species (or genotypes) as denominator. In addition, for all plant species average SIR was calculated upon addition of the first three substrates that contributed most to the separation of the plant species, based on the discriminant analysis. Data analyses was done using IBM SPSS version 19.

## 2.3 Results

### Experiment 1

Substrate-induced respiration was higher in rhizosphere samples as compared to bulk soil for five out of the eight plant species, but not for the grasses *A. odoratum*, *A. capillaris* and *H. lanatus* (Table 2.3 and Fig. 2.1a). All grasses taken together, we did not observe different SIR between rhizosphere and bulk soil (ANOVA for only grass species, $F = 1.15$, $p = 0.29$), whereas for the herbs SIR was higher in the rhizosphere as compared to bulk soil ($F = 32.45$, $p < 0.001$), suggesting different responses of soil microbes associated with different plant functional groups.

Microbial catabolic activities were affected by plant species both in rhizosphere and in bulk soil (Table 2.4). Discriminant analysis revealed that three functions with six substrates contributed to the separation of plant species microbial activity in the rhizosphere ($F = 2.14$, $p < 0.001$) (Table 2.5). The order of the substrates in Table 2.5 reflects the order in which the substrates entered the discriminant analysis, with the first substrate, phenylalanine, contributing most to the separation and arginine least. The first function of the discriminant analysis separated the rhizosphere microbial catabolic activities of *R. acetosa* from all other plant species, except for *F. rubra*, ($F = 14.85$, $p < 0.001$) (Fig. 2.2a). The second function separated *L. vulgare* from all other species ($F = 10.03$, $p < 0.001$) and the third function separated *P. lanceolata* from all plant species, except for *H. lanatus* ($F = 3.97$, $p = 0.003$). Microbial respiration in the rhizospheres of *R. acetosa*, *L. vulgare*, and *P. lanceolata* after addition of various substrates was, in general, higher as compared to the average respiration of the other species (Table 2.5).

*Table 2.3.*

The effects of plant species or genotype and soil zone (rhizosphere or bulk soil) and their interaction on substrate-induced respiration in Experiment 1 (grassland species), Experiment 2 (*Brassica juncea* genotypes) and Experiment 3 (*Solanum tuberosum* genotypes) based upon ANOVA.

| | *Experiment 1* | | *Experiment 2* | | *Experiment 3* | |
|---|---|---|---|---|---|---|
| | *F* | *p* | *F* | *p* | *F* | *P* |
| Plant species or genotypes (P) | 3.30 | **0.004** | 2.49 | 0.07 | 0.79 | 0.55 |
| Soil zone (S) | 46.01 | **<0.001** | 54.72 | **<0.001** | 57.20 | **<0.001** |
| P x S | 4.88 | **<0.001** | 1.65 | 0.19 | 0.20 | 0.93 |

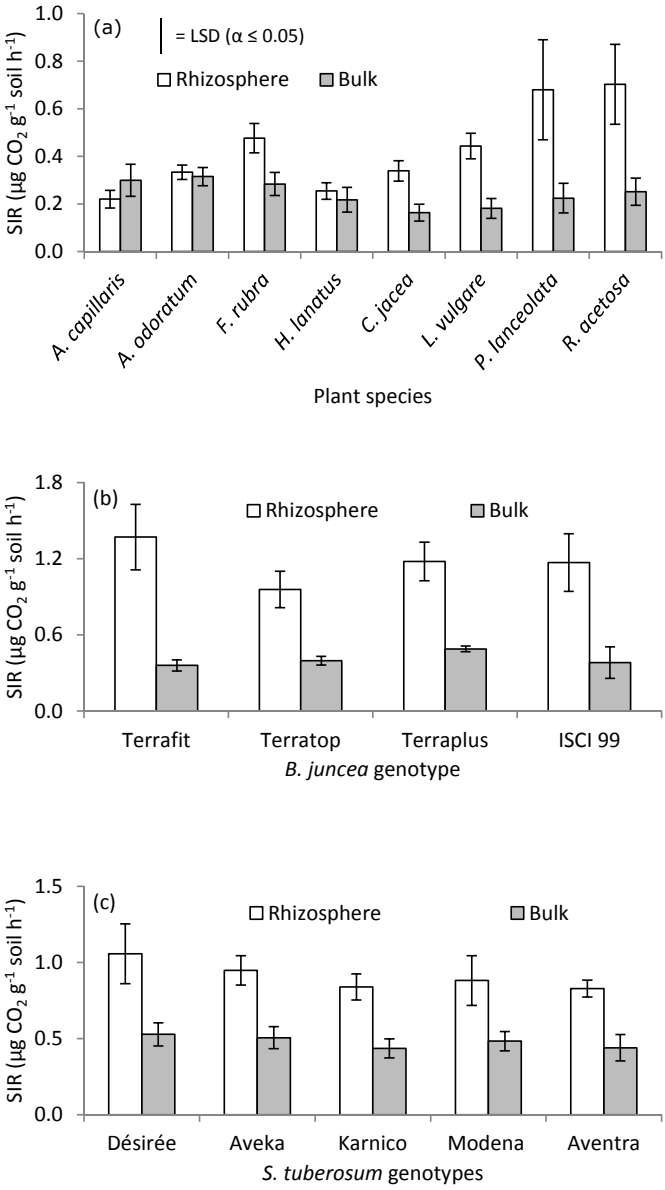


*Figure 2.1.*

Substrate-induced respiration (averaged for all substrates) in rhizosphere and bulk soil for the grassland species of Experiment 1 (a), the four grass species are on the left and the four herbs on the right, including the least significant difference (LSD), *Brassica juncea* genotypes of Experiment 2 (b) and *Solanum tuberosum* genotypes of Experiment 3 (c). Bars indicate standard errors of the mean (Experiment 1: $n = 6$, Experiment 2: $n = 4$, Experiment 3: $n = 3$).

The ratio for *L. vulgare* and *P. lanceolata* with arginine is negative (-33.23 and -8.97 respectively, Table 2.5), which in combination with a negative average arginine-induced microbial respiration implicates a higher arginine-induced microbial respiration for these species. In contrast, rhizosphere microbial respiration in *R. acetosa* was lower after addition of citric acid and arginine than that of other plant species. The three herbs were high in SIR for phenylalanine, trehalose, and fumaric acid (Table 2.5), but addition of phenylalanine also resulted in high SIR for *F. rubra* (Fig. 2.3).

In the bulk soil, microbial SIR in soil of *L. vulgare* was different from bulk soil of *A. odoratum*, *F. rubra,* and *R. acetosa* ($F = 4.67$, $p < 0.001$) (Fig. 2.2b). Sucrose-induced microbial respiration in the bulk soil of *L. vulgare* was higher, but phenylalanine-induced respiration was lower as compared to *A. odoratum*, *F. rubra* and *R. acetosa* (Table 2.5).

*Table 2.4.*

MANOVA summary based on Wilks'Lambda of the catabolic diversity of the microbial community in rhizosphereand in bulk soil samples for Experiment 1 (grassland species), Experiment 2 (*Brassica juncea* genotypes) and Experiment 3 (*Solanum tuberosum* genotypes).

| | *Experiment 1* | | *Experiment 2* | | *Experiment 3* | |
|---|---|---|---|---|---|---|
| | *F* | *p* | *F* | *p* | *F* | *p* |
| Rhizosphere | 2.14 | **<0.001** | 1.61 | 0.19 | 5.13 | **0.004** |
| Bulk soil | 1.41 | **0.037** | 0.16 | 0.58 | 0.71 | 0.76 |

*Table 2.5.*

Summary of the main findings from the microbial catabolic diversity based on the discriminant analysis, showing substrates that distinguished (per discriminant function) *Rumex acetosa*, *Leucanthemum vulgare*, and *Plantago lanceolata* from other plant species (*Agrostis capillaris, Anthoxanthum odoratum, Festuca rubra, Holcus lanatus*, *Centaurea jacea*, *L. vulgare*, *P. lanceolata*, and *R. acetosa*) due to microbial responses in rhizosphere and bulk soil of Experiment 1. Substrate-induced respiration (SIR) was averaged (± s.e.) for each substrate that contributed to the separation, the order of substrates is based on the entries of the substrates in the analysis. The ratio is based on SIR for each separated species divided by the species it was separated from.

| | Function | Separated species | Separated from | Substrate | SIR[1] (μg $CO_2$ $g^{-1}$ soil $h^{-1}$) | Ratio |
|---|---|---|---|---|---|---|
| Rhizosphere | 1 | *R. acetosa* | *A. capillaris*, *A. odoratum*, *C. jacea*, *H. lanatus*, *L. vulgare*, and *P. lanceolata* | Phenylalanine | 0.40 ±0.05 | 2.10 |
| | | | | Trehalose | 0.21 ±0.04 | 2.13 |
| | | | | Fumaric acid | 0.13 ±0.02 | 1.53 |
| | | | | Citric acid | 0.06 ±0.02 | -1.57 |
| | | | | Sucrose | 0.14 ±0.03 | 2.43 |
| | | | | Arginine | 0.00 ±0.04 | -78.04 |
| | 2 | *L. vulgare* | All species | Phenylalanine | 0.42 ±0.05 | 1.47 |
| | | | | Trehalose | 0.24 ±0.04 | 2.21 |
| | | | | Fumaric acid | 0.13 ±0.02 | 1.44 |
| | | | | Citric acid | 0.04 ±0.02 | 2.19 |
| | | | | Sucrose | 0.17 ±0.03 | 1.91 |
| | | | | Arginine | -0.01 ±0.03 | -33.23 |
| | 3 | *P. lanceolata* | *A. capillaris*, *A. odoratum*, *C. jacea*, *F. rubra*, *L. vulgare*, and *R. acetosa* | Phenylalanine | 0.44 ±0.06 | 1.40 |
| | | | | Trehalose | 0.22 ±0.04 | 2.36 |
| | | | | Fumaric acid | 0.14 ±0.02 | 1.33 |
| | | | | Citric acid | 0.04 ±0.02 | 2.39 |
| | | | | Sucrose | 0.17 ±0.03 | 1.86 |
| | | | | Arginine | -0.03 ±0.01 | -8.97 |
| Bulk | 1 | *L. vulgare* | *A. odoratum, F. rubra,* and *R. acetosa* | Sucrose | -0.06 ±0.04 | -1.95 |
| | | | | Phenylalanine | 0.30 ±0.04 | 0.36 |

[1] Average substrate-induced respiration with standard errors. For rhizosphere, n=33 for function 1, n=44 for function 2, and n=34 for function 3; for bulk, n=18 for function 1.

## *Experiment 2*

Average SIR was higher in rhizosphere samples as compared to bulk soil for all *B. juncea* genotypes (Fig 2.1b). Within the rhizosphere or bulk soil, the microbial catabolic diversity did not vary among *B. juncea* genotypes (Table 2.4).

## *Experiment 3*

Also in the potato genotype experiment, average SIR was higher in rhizosphere samples than in bulk soil samples for all genotypes (Fig 2.1c). Furthermore, the catabolic diversity of the microbial community was affected by potato genotype in the rhizosphere but not in the bulk soil (Table 2.4). Using the stepwise approach of the discriminant analysis, no differences between the genotypes was detected. However, without using the stepwise approach the discriminant analysis revealed that genotypes Aveka and Modena could be separated from Aventra, Désirée and Karnico based on one discriminant function. Respiration upon the addition of sucrose and trehalose contributed most to the separation, based on the canonical variate correlation coefficients. Analysis of the discriminant function scores confirmed the separation between the genotypes ($F = 31.91$, $p < 0.001$). No differences were observed in microbial responses upon addition of substrates in bulk soil of the potato genotypes.

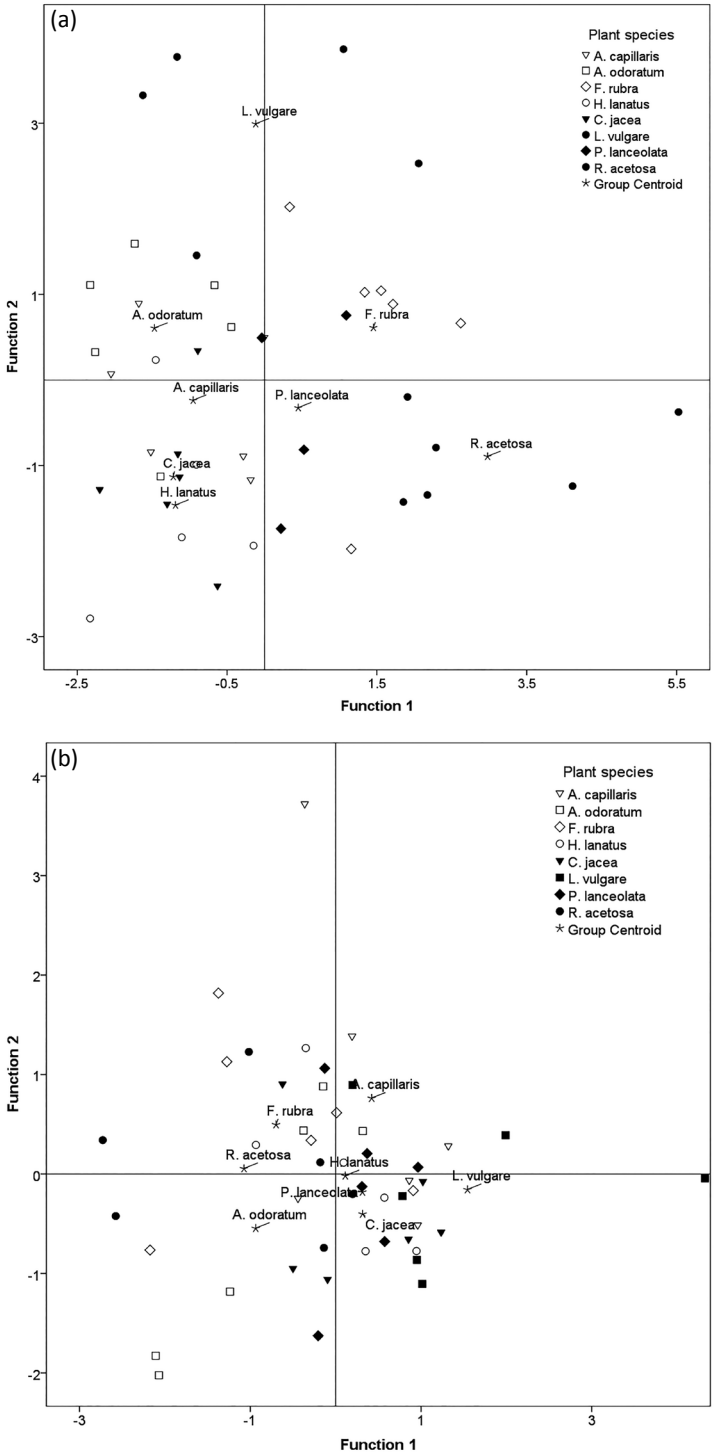


*Figure 2.2.*

Scatter plots of the microbial catabolic diversity in the rhizosphere (a) and bulk soil (b) of plant species in Experiment 1 as indicated by the first two discriminant functions. Asterisks show plant species centroids, closed and open symbols show single values of each plant species.

## 2.4 Discussion

### *Discrimination between rhizosphere and bulk soil using the MicroResp™*

Microbial SIR was higher in the rhizosphere than in bulk soil for all genotypes of *B. juncea* and *S. tuberosum* and for the majority of the grassland species tested. Easily available C in plant root exudates results in microbial communities with a higher potential to rapidly decompose added substrates in the rhizosphere as compared to the bulk soil (Blagodatskaya *et al.*, 2010 & Oger *et al.*, 2004). Our study confirms that MicroResp™ is able to detect higher microbial SIR in the rhizosphere compared to bulk soil. Higher activities measured are a reflection of the microorganisms actively consuming comparable substrates *in situ* at the time of sampling, for example in the form of root exudates. Similar SIR in rhizosphere and bulk soil was observed for the grasses *Agrostis odoratum*, *Anthoxanthum capillaris* and *Holcus lanatus*. Most likely, these similarities between the soil zones are related to a more dense root system of grasses in the sampled layer as compared to the herbs (Kutschera and Lichtenegger, 1982 & 1992). This is also supported by the findings that grass species had more root biomass than the herbs in the sampled layers (Mommer *et al.*, 2010).

### *Plant species and genotype rhizosphere effects*

Microbial catabolic activities in the rhizosphere of the herbs *R. acetosa*, *L. vulgare* and *P. lanceolata* differed from the other species due to higher respiration on a set of six substrates, i.e. two amino acids, two organic acids and two carbohydrates. This suggests that microbes in the rhizosphere of the three herbs are more adapted to these substrates as the other plant species, which could possibly be caused by higher rates of such substrates in the rhizodeposits. These substrates are also the major types of C rich root exudates (Dakora and Phillips, 2002). Phenylalanine in the MicroResp™ had the strongest effect on the separation of the plant species. If such an effect is caused by higher levels in root exudates of these species, than it is remarkable that the herbs *R. acetosa*, *L. vulgare* and *P. lanceolata* resulted in a comparable SIR response upon addition of phenylalanine (Fig. 2.3), whereas the grasses did not. The four grasses are Poaceae, whereas *R. acetosa* belongs to the Polygonaceae, *P. lanceolata* to the Plantaginaceae and *L. vulgare* to the Asteraceae. So, the phylogenetic distance is larger among the herbs as compared to the grasses investigated. The findings presented here suggest that less related species (increasing plant phylogenetic diversity) can have similar belowground effects. Confirmation on the composition of root exudates for the species tested is necessary, but our MicroResp™ findings can be helpful to explore linkages between ecosystem functioning and phylogenetic diversity further (e.g. Srivastava *et al.*, 2012 & Navarro-Cano *et al.*, 2014).

Besides plant species specific effects, we also observed genotypic effects in the microbial catabolic activities in the rhizosphere of potato genotypes. The rhizosphere's microbial respiration upon addition of C rich substrates in potato genotypes Aveka and Modena was lower than that of Aventra, Désirée and Karnico. In a recent study, Dias *et al.* (2013) observed that the relative proportions of rhizosphere gram-negative bacteria *Pseudomonas* and *Burkholderia* spp. differed between Modena and Karnico which could have been caused by root exudates. However, root exudation patterns of these genotypes have not been determined and future studies investigating potato rhizodeposits could reveal possible variations in their profiles.

The number and type of substrates included in the MicroResp™ system might have contributed to the finding that no *B. juncea* genotype effects were observed. On the other hand, the potato genotype experiment showed that by using the same set of substrates it was possible to detect genotypic effects in the microbial response. Similarly, Stevenson *et al.* (2004) reported that a set of two substrates was equally effective at differentiating the microbial catabolic response as the entire set of substrates. In their study, the substrates glucose and α-ketobutyric acid could classify pasture versus forest sites and explained 95% of the variance. However, genotypic differences between plants growing in one and the same site can be expected to be more refined than differences between ecosystem types.

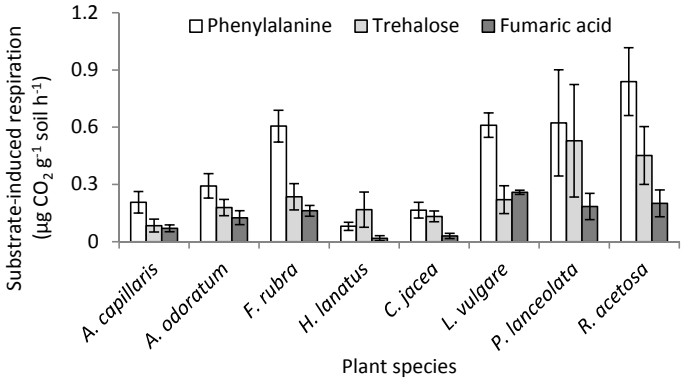


*Figure 2.3.*

Average substrate-induced respiration upon addition of phenylalanine, trehalose and fumaric acid for each plant species in Experiment 1. Error bars are standard errors of the mean with $n$ = 6.

### *Beyond rhizosphere effects*

We have demonstrated that plant species can affect the soil beyond what we defined as the rhizosphere; the microbial catabolic diversity in the bulk soil of *L. vulgare* was different from *A. odoratum*, *F. rubra* and *R. acetosa*. In the rhizosphere, the microbial catabolic diversity of *L. vulgare* differed from all other seven species tested. This indicates that *L. vulgare* induced effects were more prominent in the rhizosphere and extended into the bulk soil. Similarities among the distinguishing substrates in the two soil zones of *L. vulgare* supported a functional linkage between rhizosphere and bulk soil (Table 5). Similarly, plant-feedback effects were observed in soils that were previously grown by *L. vulgare* (Hendriks *et al.*, 2013). Soils that were conditioned by growing *L. vulgare* resulted in higher biomass of the follow-up plant species and it was suggested by the authors that pathogenic microorganisms were causing these legacy effects. In addition, such belowground effects of *L. vulgare* were not detected in soils that were previously grown by *A. odoratum*, *F. rubra* or *P. lanceolata*.

## *2.5 Conclusions*

We were able to separate plant species and potato genotypes by means of the microbial catabolic response in the rhizosphere and plant species-induced effects were also detected in bulk soil. Maintaining monospecies plots for more than 10 years of weeding resulted in clear differences in the microbial catabolic diversity in these two soil zones. The selection of substrates in the MicroResp™ detection system does not only indicate experimental treatment effects, but has the potential to link observed plant-induced effects to ecosystem functioning. Future studies using MicroResp™ could include C rich substrates that contain elements such as phosphorus to add a more in depth understanding of plant-interactions.

# Chapter 3

## *In situ* effects of root sinigrin on the rhizobiome

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## *Abstract*

Brassicaceae are known to produce glucosinolates, which can be hydrolysed into isothiocyanates that are important biocontrol agents. This study examines the effect of root 2-propenyl glucosinolate (sinigrin) on rhizosphere biota of *Brassica juncea* (Indian mustard). We hypothesized that increasing root glucosinolate concentrations (here: sinigrin) of *B. juncea* genotypes would affect the microbial community and consequently the nematode community in the rhizosphere. We used four *B. juncea* genotypes with a range in root sinigrin concentrations, the main glucosinolate produced by *B. juncea*. The microbial catabolic diversity was characterized via the MicroResp™ system and nematode community composition was assessed by quantitative PCR. Root sinigrin concentration significantly correlated with an increased microbial community's respiration ($p$ = 0.02) and with the total number of nematodes ($p < 0.05$), while no relationships were observed with the composition of the nematode community. These results support a role of secondary plant metabolites in the composition of the rhizobiome, as root sinigrin affects microbial community functioning.

## 3.1 Introduction

The rhizosphere is densely populated by a diverse microbial and faunal community. On average, as much as 25% of the photosynthetically-assimilated carbon (C) is deposited in the rhizosphere (Jones *et al.*, 2009). Root exudates consist of many different C based components which can stimulate or suppress population growth and activity within the soil biota, with potential effects on ecosystem functioning (Cheng *et al.*, 2014). It has been suggested that plant genotypes also influence their microbial rhizosphere, probably as a result of the composition of root exudates (Badri *et al.*, 2013 & Sugiyama *et al.*, 2013). Root exudates of crucifers belonging to the genus *Brassica* have been widely investigated, both with empirical and simulated data (e.g. Bekaert *et al.*, 2012 & Lankau, 2007).

Different genotypes of *Brassica juncea* are known to vary in root composition, including glucosinolate concentration (Bellostas *et al.*, 2007 & Schütze *et al.*, 2004). Glucosinolates can be hydrolysed by the enzyme myrosinase into compounds, such as isothiocyanates, that are toxic to a broad range of organisms (Morra and Kirkegaard, 2002). Isothiocyanate toxicity is most likely due to an irreversible and nonspecific reaction with amino acids and proteins (e.g. Brown and Morra, 1997). Glucosinolates are located in the outer periderm layer of the root (McCully *et al.*, 2008) and glucosinolates, isothiocyanates and myrosinases have all been detected in the rhizosphere of Brassicaceae (Borek *et al.*, 1996 & Rumberger and Marschner, 2003). Root glucosinolate concentration is likely to contribute to the prevention of pathogen infection belowground (McCully *et al.*, 2008 & Rumberger and Marschner, 2004 & Schreiner and Koide, 1992). However, also non-pathogenic soil organisms are likely to be affected by the release of glucosinolates and isothiocyanates (Smith and Kirkegaard, 2002). One of the main glucosinolates in *B. juncea* is 2-propenyl glucosinolate (sinigrin), which is hydrolysed into 2-propenyl (allyl) isothiocyanate (Olivier *et al.*, 1999). *In vitro* experiments have shown that allyl isothiocyanate is toxic to microorganisms (Manici *et al.*, 1997 & Sarwar *et al.*, 1998) and nematodes (Donkin *et al.*, 1995 & Lazzeri *et al.*, 2004) at concentrations that could potentially be reached during crop growth (Morra and Kirkegaard, 2002).

Here, we report on the *in situ* effects of root sinigrin concentration on the rhizobiome of Brassicaceae. To induce a large range in sinigrin concentration during a growing season, we used four *Brassica* genotypes and the grass *Triticum aestivum* L. as control. We hypothesized that increasing root sinigrin concentration would have a negative effect on the microbial rhizosphere community, resulting in a reduction in respiration and a change in the microbial community's catabolic response. We further hypothesized that root sinigrin effects on the microbial community should be reflected in the nematode community, as the abundance of the

microbivorous nematodes would be indirectly affected due to the sinigrin induced effects on the microbial community (Ferris *et al.*, 2004). In addition, direct effects due to toxicity of hydrolysed isothiocyanates on nematodes were expected.

## *3.2 Material and Methods*

### *Site description and environmental conditions*

The field experiment was conducted in 2010 in Münster, Germany. The soil texture was sandy with 77.2% sand, 13.6% silt, and 9.2% clay; soil pH was 6.4 in $CaCl_2$, soil organic matter content 1.3%, total nitrogen (N) 0.09%, total phosphorus (P) 0.14% and total potassium (K) 0.02%. The previous crop (*Zea mays* L.) was about 70 cm tall when it was incorporated into the soil two days before sowing. Additional fertiliser of 70 kg N $ha^{-1}$ and 17.5 kg sulphur (S) $ha^{-1}$ was applied at the day of sowing.

Soil temperature was recorded hourly throughout the experiment at 10 cm depth. Precipitation was measured daily with a pluviometer on the field. The soil temperature dropped promptly in July, increased shortly thereafter and decreased gradually towards the end of the growing season. Average soil temperature for the growing period was 18.8°C (±3.8). Precipitation was regular in July, while in August several peaks in precipitation occurred and a very high peak was observed at the end of August, with almost 90 mm precipitation in a single day (Fig. 3.1).

### *Experimental design*

Four *B. juncea* genotypes with different glucosinolate concentrations and flowering times were selected, namely Terrafit, Terratop and Terraplus (P.H. Petersen in Lundsgaard, Germany) and ISCI 99 (Research Institute for Industrial Crops in Bologna, Italy). *Triticum aestivum* (cv. Hermann) was selected as a non-glucosinolate producing control crop. Seeds were sown on July 9$^{th}$, 2010, in plots of 4 × 15 m using a complete randomized block design with four replications. Seed densities were 12 kg $ha^{-1}$ for Terrafit, Terratop and Terraplus, 15 kg $ha^{-1}$ for ISCI 99, and 176 kg $ha^{-1}$ for *T. aestivum*.

## *Plant and rhizosphere sampling*

Rhizosphere biota and plant roots were sampled at 32, 52, and 59 days after sowing. Plant root systems (50 × 50 cm soil surface area) were excavated carefully by using a spade to ensure sampling of intact root systems. Root systems and adhering soil were shaken twice and the adhering soil, regarded as rhizosphere soil, was removed from the roots using a brush and immediately stored at 4°C. After removal of adhering soil, the root dry weight was measured after oven drying (70°C, 24 h).

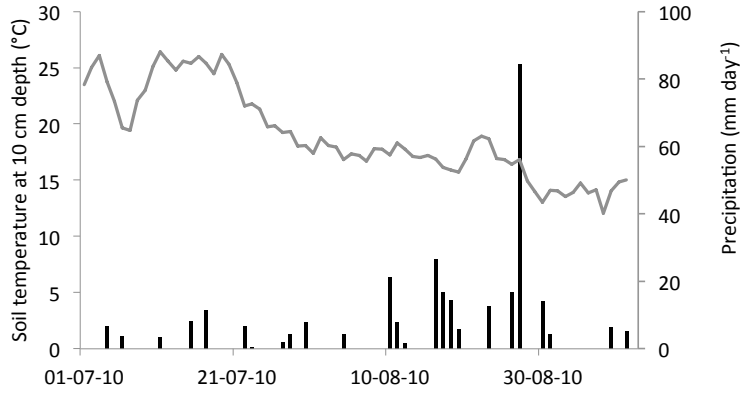


*Figure 3.1.*

Daily soil temperature (°C) measured at 10 cm depth and precipitation (mm day$^{-1}$) measured with a pluviometer during the experimental growing season.

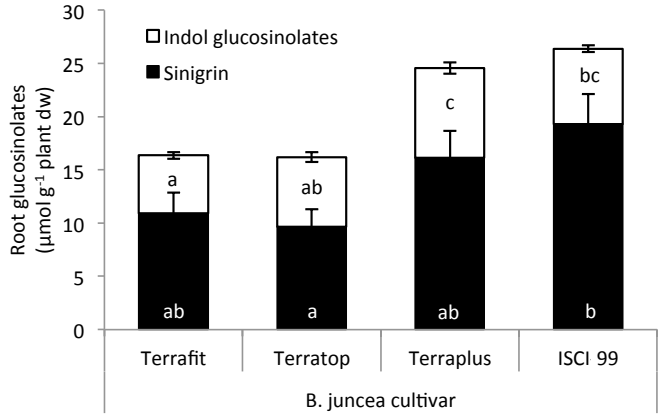


*Figure 3.2.*

Average root sinigrin and indol glucosinolate concentration for the *Brassica juncea* genotypes Terrafit, Terratop, Terraplus and ISCI 99 over the growing period. Different letters indicate differences between genotypes (post-hoc Tukey HSD). Bars show standard errors of the mean ($n$ = 12).

For glucosinolate analysis, 10 *Brassica* plants per plot were randomly selected at each sample occasion. After removing carefully the soil, all the root material was freeze-dried and milled using an oscillating mill (MM2, Retsch Germany). Sinigrin and indol glucosinolate concentrations were determined using HPLC (series 1100, Agilent DAD) after extraction with methanol and water (70/30, v/v) (Schütze *et al.*, 2004). Indol glucosinolate concentration was comprised of glucobrassisin, 4-methoxy glucobrassisin, 4-hydroxy glucobrassisin and neo-glucobrassisin. All glucosinolate concentrations were expressed as µmol $g^{-1}$ root dry weight for each plot and sample occasion.

## *Microbial catabolic diversity*

The MicroResp™ system (Campbell *et al.*, 2003) was used to measure the microbial catabolic activities. Substrates used were glucose, sucrose, trehalose, malic acid and tween-80, with water as control. Substrate selection was based on root exudates (Campbell *et al.*, 1997) and in addition a more recalcitrant substrate was included (tween-80). Substrates were dissolved in demineralised water and prepared as stock solutions designed to deliver 30 mg C $g^{-1}$ soil solution after dispensing 25 µl of the solution. Each substrate was dispensed into four replicate wells of a deepwell plate (1.2 ml, Fischer Scientific, Germany). The rhizosphere soil samples were sieved (2 mm mesh) and added directly to the deepwells using the standard filling device (see Campbell *et al.*, 2003), adding 300 µl soil to each well. Samples were not pre-incubated, because plant induced rhizosphere effects might be lost during 48 h of pre-incubation (Jones and Darrah, 1993). The moisture content of the soil samples was determined separately (105°C for 24 h). The fresh soil weight added to each deepwell plate was measured and used to calculate the average weight per well per sample. Absorbance of the indicator dye was measured in the detection plate at 570 nm before and after the incubation period using a micro plate reader (VMAX, Molecular Devices). Colour development was normalized by subtracting the averaged time zero measurements for each plate from the measured colour development per well after 6 h incubation according to Campbell *et al.* (2003) and converted to $CO_2$ concentrations using a calibration curve $\%CO_2 = 0.02 \times A_{570}^{-3.11}$ ($R^2 = 0.93$). Where $\%CO_2$ (v/v) is the concentration in the headspace after incubation and $A_{570}$ is the normalized absorbance. Median $CO_2$ concentrations ($n = 4$) for each substrate per plot were converted to respiration rates (µg $CO_2$–C $g^{-1}$ soil $h^{-1}$) using soil fresh weight and soil water content. Median respiration rates of the control, i.e. water only, were subtracted from the median respiration rates induced by a C source. The calibration curve for absorbance ($A_{570}$) *versus* headspace $CO_2$ concentrations was determined by placing strips from breakable strip plates in airtight bottles with $CO_2$ concentrations ranging from 0.02-1.5%. After 6 h incubation $CO_2$ inside the bottle was measured by gas chromatography (Finnigan TraceGC, Interscience) and the strips were measured using the microplate reader.

## *Nematode community analysis*

Nematodes were extracted, per plot and at each sample occasion, from 30 g of rhizosphere soil by mixing the soil with 2 L of water. The solution was placed directly onto a double cotton wool filter (200 mm diameter, Favorit Universal; NIFA Technologies) which was placed in an extraction dish filled with tap water. After two days on the filter, all the nematodes that had moved through the cotton wool filter into the extraction dish were collected in 100 ml water. For the suspensions, nematode density was estimated for all plots and at each sampling occasion by counting two subsamples of 5 ml under a low magnification light microscope (magnification 40x), after which the subsamples were poured back into the original suspension. Subsequently, DNA was extracted from the suspensions and purified as described by Vervoort *et al.* (2012). Based on microscopic nematode biodiversity assessment and primer availability ten taxa were selected for qPCR for the nematode community composition (Table 3.1). Cluster-specific primers D1 and M3 were used for the suborder Dorylaimina and the family Mononchidae (Holterman *et al.*, 2008). For the family Plectidae, separate primers were used that either targeted *Anaplectus* or *Plectus* (the only two representative genera of this family in our field experiment).

*Table 3.1.*

Average nematode densities (# 100 $g^{-1}$ soil) (± standard errors; $n$ = 20) for the whole community (counted microscopic samples) and for ten selected nematode taxa (quantitative PCR analysis) per sample time.

| | Functional group[a] | Days after sowing | | | | | |
|---|---|---|---|---|---|---|---|
| | | 32 | | 52 | | 59 | |
| Whole community | - | 6772 | ±817 | 8925 | ±2100 | 7713 | ±1238 |
| Achromadoridae | O | 96 | ±47 | 577 | ±252 | 438 | ±220 |
| *Anaplectus* | B | 60 | ±19 | 21 | ±7 | 19 | ±8 |
| Aphelenchidae | F | 39 | ±12 | 7 | ±2 | 36 | ±14 |
| Cephalobidae | B | 1950 | ±424 | 1541 | ±348 | 1150 | ±275 |
| Dorylaimidae D1 | O | 10 | ±7 | 17 | ±6 | 5 | ±3 |
| *Mesorhabditis* | B | 208 | ±61 | 50 | ±12 | 47 | ±13 |
| Monhysteridae | B | 226 | ±63 | 1690 | ±501 | 609 | ±122 |
| Mononchidae M3 | C | 23 | ±13 | 1297 | ±493 | 140 | ±47 |
| Panagrolaimidae | B | 77 | ±28 | 28 | ±18 | 81 | ±46 |
| *Plectus* | B | 6 | ±4 | 31 | ±20 | 110 | ±74 |

[a] Functional groups are bacterivorous (B), carnivorous (C), fungivorous (F), and omnivorous (O) nematodes.

## *Data analyses*

Data of root glucosinolate concentration, nematode density and the nematode community composition were $^{10}$log-transformed to meet the assumptions of normality and homogeneity of variances for the residuals.

Effects of genotype, time of sampling and their interaction were tested on levels of sinigrin and indol root glucosinolate, and nematode density by using ANOVA (SPSS IBM; version 19). When an effect was observed, Tukey HSD test was applied to differentiate between treatments.

Principal component analysis (PCA) (CANOCO 5.0) was used to calculate case scores of the first axis of the microbial catabolic diversity and of the nematode community composition (cf. Van Dobben *et al.*, 1999). The first axis of the microbial PCA explained 73% of total variation and the first axis of the nematode community PCA explained 41% of total variation. Pearson's correlation coefficients were calculated to determine linkages between the nematode density, root glucosinolates, the case scores of the first axis of the microbial PCA, and the case scores of the first axis of the nematode community PCA.

Effects of genotype, time of sampling and their interaction were tested on the microbial catabolic diversity and the nematode community composition through Monte Carlo permutation tests by redundancy analysis (RDA) (CANOCO 5.0) (Van Dobben *et al.*, 1999). Since an effect of time was observed in microbial catabolic diversity and nematode community composition, we included root sinigrin and indol glucosinolate concentrations, root biomass, soil temperature and soil moisture as explanatory variables. Soil temperature was averaged for the 20 days preceding sampling. For the RDA of the nematode community composition, the case scores of the first axis of the microbial PCA were also included. Forward selection, i.e. stepwise selection of explanatory variables, was used to select for variables comprising the strongest effects and sort-out variance that already was explained by a former variable. Monte Carlo permutation tests (999 random permutations) were performed to indicate effects of explanatory variables (sinigrin, indol glucosinolates, root biomass, soil temperature and soil moisture) on the dependent variables (microbial catabolic diversity and nematode community composition). Block effects were partialled out by entering block indicator variables as covariables. Explanatory variables that comprised significant associations ($p < 0.05$) to the microbial activities or to the nematode community composition were included in ordination plots.

## 3.3 Results

### Glucosinolates

*Brassica* genotypes varied significantly in their root sinigrin and indol concentrations (Table 3.2). Total glucosinolates analysed were higher in genotypes Terraplus and ISCI 99 as compared to genotypes Terrafit and Terratop (Fig. 3.2). Root sinigrin concentration was the highest in ISCI 99 and the lowest in Terratop. Root indol concentration was the highest in Terraplus and the lowest in Terrafit.

### Microbial catabolic diversity

The microbial response upon addition of C rich substrates was affected by time (21.2% explained variance, *F*-ratio = 7.8, $p < 0.001$, based on RDA), but not by genotype and no interaction between time and genotype was observed. In the follow up analysis, the microbial catabolic diversity was affected by root sinigrin concentration and soil temperature (Table 3.3). Root sinigrin concentration showed associations with induced microbial respiration upon the addition of sucrose and malic acid (Fig. 3.3a). Negative associations were observed between soil temperature and the substrates used in the MicroResp™.

*Table 3.2.*

The effects of genotype, sampling time and the interaction between genotype and sampling time on root sinigrin concentration (µmol $g^{-1}$ root), root indol glucosinolates (µmol $g^{-1}$ root) and nematode density in the rhizosphere (# 100 $g^{-1}$ soil), based on 2-way ANOVA (significant values in bold; $n = 4$).

| | Root sinigrin[a] | | Root indol glucosinolates[a] | | Nematode density[b] | |
|---|---|---|---|---|---|---|
| | *F* | *p* | *F* | *p* | *F* | *p* |
| Genotype (G) | 3.04 | **0.043** | 13.49 | **<0.001** | 2.80 | **0.038** |
| Time (T) | 1.49 | 0.241 | 11.54 | **<0.001** | 0.07 | 0.931 |
| G × T | 0.59 | 0.736 | 0.91 | 0.503 | 1.19 | 0.329 |

[a] analysis without control plots
[b] analysis with control plots included

## *Total nematodes*

The nematode density did not differ among the *Brassica* genotypes and was high with a mean of 8695 ±1020 nematodes 100 $g^{-1}$ dry soil for all Brassicaceae. In comparison with the *Brassica* genotypes, nematode density was relatively low in the control plots (*T. aestivum*) with a mean of 4236 ± 370 nematodes 100 $g^{-1}$ dry soil. Nematode density was positively correlated with root sinigrin (Pearson's $r = 0.26$, $p < 0.05$, $n = 60$), root indol glucosinolates ($r = 0.30$, $p = 0.02$, $n = 60$) and with the case scores of the first axis of the microbial PCA ($r = 0.30$, $p = 0.02$, $n = 60$).

## *Nematode community*

Similar to the microbial catabolic diversity, the nematode community composition was only affected by time (19.3% explained variance, *F*-ratio = 6.9, $p < 0.001$, RDA). In the follow up analysis, no associations were observed between root glucosinolate concentration and nematode genera or families. Furthermore, no correlations were observed between the catabolic activities of the microbial community and nematode community composition. The rhizosphere nematode community composition was affected by soil temperature and soil moisture (Table 3.3). The nematode taxa Dorylaimidae D1 and Mononchidae M3 showed positive associations with soil moisture, while *Anaplectus* and *Mesorhabditis* showed positive associations with soil temperature (Fig. 3.3b).

*Table 3.3.*

The effects (variance explained, pseudo-*F* and *p*-values) of sinigrin and indol glucosinolate concentrations (µmol $g^{-1}$ root), root biomass (g $m^{-2}$), soil temperature (°C) and soil moisture (%), on the microbial community catabolic diversity (based on 5 substrates) and the nematode community composition (based on 10 nematode taxa), based on RDA (significant values in bold).

| | Microbial catabolic diversity | | | Nematode community composition | | |
|---|---|---|---|---|---|---|
| | Var (%) | *F* | *p* | Var (%) | *F* | *p* |
| Sinigrin | 6.0 | 4.4 | **0.02** | 0.9 | 0.6 | 0.73 |
| Indol glucosinolates | 1.2 | 0.9 | 0.39 | 1.1 | 0.7 | 0.62 |
| Root biomass | 0.8 | 0.6 | 0.52 | 0.6 | 0.4 | 0.84 |
| Soil temperature | 18.9 | 13.3 | **0.002** | 4.9 | 3.1 | **0.02** |
| Soil moisture | 1.6 | 1.2 | 0.19 | 8.3 | 5.2 | **0.001** |
| MA-PCA[a] | - | - | - | 1.7 | 1.1 | 0.30 |

[a] Case scores of the microbial catabolic diversity PCA were only included in the nematode community composition

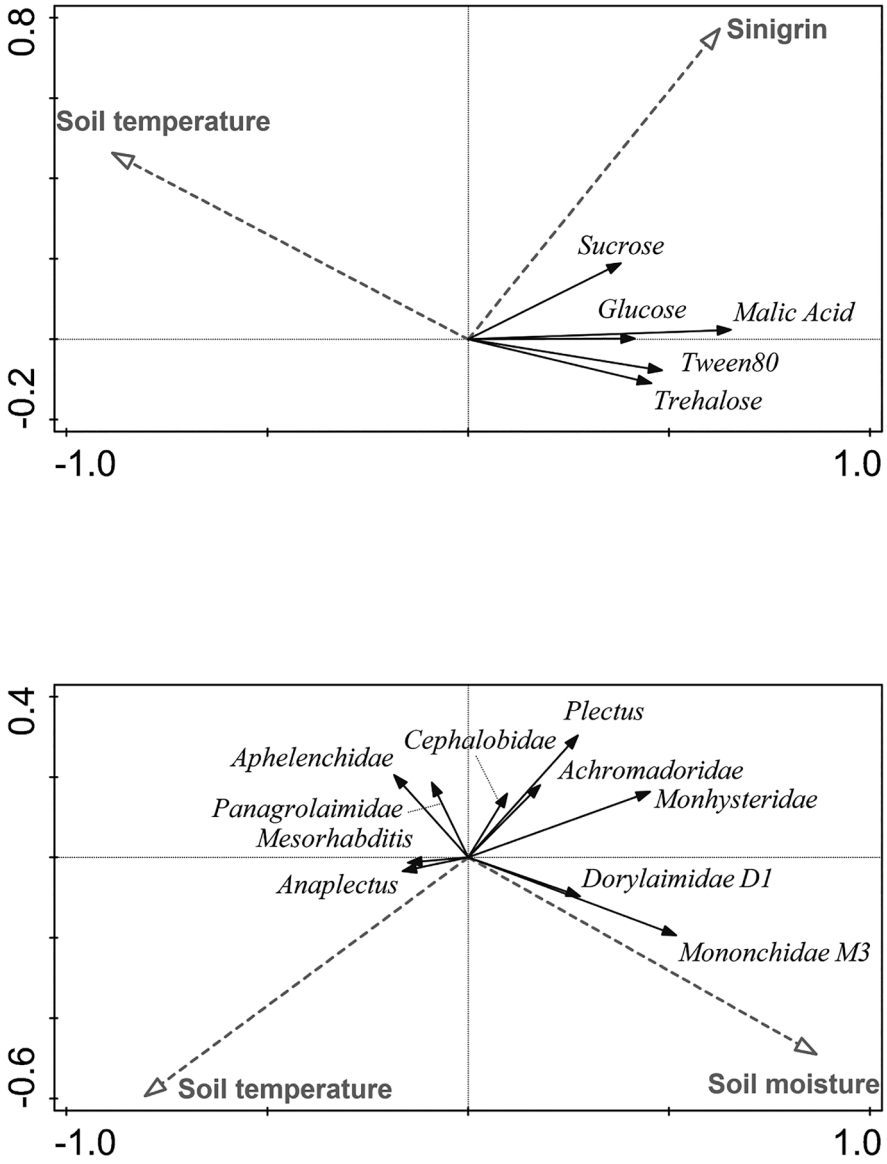


*Figure 3.3.*

Ordination diagram of the microbial catabolic diversity (a) based on RDA with root glucosinolate concentration and the soil temperature accounting for 24.9% of the variance. Ordination diagram of the nematode community composition (b) based on RDA with soil temperature and soil moisture accounting for 13.2% of the variance.

## 3.4 Discussion

We observed that higher root sinigrin concentrations in *B. juncea* increased the catabolic activity of the microorganisms and enhanced the nematode density in the rhizosphere, which was in contrast to what we expected. In addition, the catabolic activity of the microorganisms was positively correlated with the nematode density.

Sinigrin concentrations measured in the *B. juncea* roots in this study were comparable to previously reported concentrations (Kirkegaard and Sarwar 1998 & Sarwar and Kirkegaard 1998). We expected a negative effect of increasing root sinigrin concentration on rhizobiota, assuming sinigrin was hydrolysed into toxic allyl isothiocyanate in the rhizosphere. The fact that no negative effect occurred indicates that sinigrin was either not hydrolysed, or the compounds formed after hydrolysis of sinigrin stimulated organisms in the rhizosphere. Similar effects have been observed by Zeng *et al.* (2003), who found growth stimulation of ectomycorrhizal fungi by root exudates of many crucifers, and stimulation was even enhanced by addition of myrosinases. Moreover, stimulating effects of allyl isothiocyanate have been observed in oxidative-stress resistance of *Caenorhabditis elegans* (Hasegawa *et al.*, 2010). On the other hand, no changes in plant-parasitic and free-living nematodes could be attributed to the release of allyl isothiocyanate in the bulk soil (Vervoort *et al.*, 2014). Hydrolysation of glucosinolates results in isothiocyanate, thiocyanate, nitriles, epithionitrile and oxazolidine-2-thiones, depending on conditions such as organic matter, temperature, pH and the presence of metal ions in the rhizosphere, all with different potential impacts on the rhizobiome (Wittstock and Halkier, 2002). Kong *et al.* (2012) for example found that with decreasing rhizosphere pH, glucosinolate hydrolysation resulted in more nitriles and epithionitriles in the presence of Fe(II)-ions and epithio-specifier and nitrile-specifier proteins. That such changes can result in shifts in the microbial rhizosphere community was shown by Buxdorf *et al.* (2013) who observed that the fungus *Alternaria brassicicola* had a preference for nitrile-producing hosts in a study on other crucifers, namely with *Arabidopsis thaliana* mutants.

The catabolic activity of the microbial community correlated positively with nematode density in the rhizosphere. Correlations between activity of microbes and nematode density can theoretically be explained via two mechanisms. An increase in nematodes can be due to increased activity of the microbial community. Opportunistic bacterivorous nematodes can increase rapidly when sufficient resources become available. Nevertheless, we did not observe any correlation between the analysed bacterivorous nematode taxa and the catabolic activity of the microbial community. The microbial community may have adjusted faster to changing environmental conditions than the nematode community (Vonk and Mulder, 2013). Alternatively, the increase in rhizosphere

nematodes resulted in a stimulation of the microbial activity. The increase in microbial activity could be caused by leakage of C and other nutrients from root cells damaged by nematodes, as was reported by Tu *et al.* (2003). In addition, it was found that feeding of nematodes on clover roots increased the amount of photosynthetically derived C in microbes (Yeates *et al*., 1999). Furthermore, Tu *et al.* (2003) argued that plant-parasitic nematodes degrade cellulose into more labile compounds, which may have increased the overall decomposability of root detritus and leading to an increase in microbial activity.

Here we have shown that root sinigrin concentration is associated with the catabolic activity of the microbial community upon the addition of a small set of substrates as measured by MicroResp™. Changes in the microbial catabolic diversity could indicate a shift in the functioning, or a shift in the structure of the community. Rumberger and Marschner (2003, 2004) found an alteration in the composition of the bacterial community based on DGGE that was correlated with rhizosphere isothiocyanates. Similar alterations in the community composition may have occurred in our study. However, it is not possible to differentiate between microbial functioning and structure based only on the results obtained in our experiment.

## *3.5 Conclusions*

The results of this experiment showed the effects of the plant root composition on the microbial catabolic diversity and nematode density in the rhizosphere. The plant-induced effects on the microbial community were not reflected in the structure of the rhizosphere nematode community based on the ten nematode taxa investigated here. Small advantages caused by glucosinolates and hydrolysed products could alter species competition, select beneficial organisms and fend off pathogens in the rhizosphere to provide growth advantages to plants. Future studies could focus on plant-induced factors causing differences in the rhizosphere concentrations of glucosinolates and in particular the various hydrolysed compounds that it can be produced. Understanding plant-induced factors of rhizodeposition and the effects on the rhizobiome will help us further to understand rhizosphere ecology with potential benefits in terms of pest control and crop productivity.

# Chapter 4

## Hatching of *Globodera pallida* is inhibited by allyl isothiocyanate *in vitro* but not by incorporation of *Brassica juncea* tissue in soil

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## *Abstract*

The aim of this study was to assess the feasibility of controlling the potato cyst nematode *Globodera pallida* through biofumigation with glucosinolate rich *Brassica juncea* genotypes. The main glucosinolate of *B. juncea* is 2-propenyl glucosinolate (sinigrin) which is the precursor of 2-propenyl (allyl) isothiocyanate. Toxicity of allyl isothiocyanate to encysted *G. pallida* was tested *in vitro*. Fifty percent reduction in hatching was found within 2 h of exposure to 0.002% allyl isothiocyanate. Based on the *in vitro* results we hypothesized that biofumigation with *B. juncea* would reduce hatching of *G. pallida in vivo* and higher sinigrin levels would have a stronger effect. Plant genotype, sulphur fertilization and insect herbivory affected sinigrin concentration of *B. juncea*. However, increasing sinigrin concentration of *B. juncea* did not affect *G. pallida* hatching after biofumigation. The absence of an biofumigation effect was most likely due to lower concentrations of allyl isothiocyanate *in vivo* compared to *in vitro* conditions. These results show that it is difficult to reach sufficiently high levels of toxicity to reduce hatching of *G. pallida* under realistic conditions.

## 4.1 Introduction

One of the major causes of yield reduction in potato cropping is damage caused by potato cyst nematodes (*Globodera pallida* and *G. rostochiensis*) (Evans and Stone, 1977 & Trudgill, 1986). Nematode infestation by *Globodera* spp. can reduce potato yield up to 80% (Singh *et al.*, 2013). Juveniles of *Globodera* spp. invade potato roots, start feeding, and after the final moult produce eggs which remain inside their body. A female body swells and toughens, thereby forming a protective cyst. The juveniles inside the egg within the newly formed cyst are in diapause and hatch after exposure to specific root exudates. Even after many years in soil in the absence of host plants, cysts can contain viable eggs. Potato cyst nematodes can be controlled by crop rotation, application of nematicides, growth of resistant potato cultivars, or biofumigation (Been and Schomaker, 1999 & Evans and Stone, 1977 & Kerry *et al.*, 2003 & Lord *et al.*, 2011).

Biofumigation is the incorporation of glucosinolate rich plant material into the soil (Kirkegaard and Sarwar, 1998 & Van Dam *et al.*, 2009). Glucosinolates are sulphur (S) containing secondary metabolites of Brassicaceae that are involved in plant defence (Fahey *et al.*, 2001). Upon tissue disruption, for example by insect herbivory, glucosinolates come into contact with the enzyme myrosinase and as a result toxic isothiocyanates are released (Ahuja *et al.*, 2010). The release of isothiocyanate is the basis of biofumigation, although tissue disruption is achieved with machines rather than through herbivory (Morra and Kirkegaard, 2002).

The life cycles of potato crops and cyst nematodes are synchronized, second stage juveniles will only hatch from encysted eggs in the presence of root exudates of a suitable host. During all active life stages, potato cyst nematodes live in close contact with potato roots. Since biofumigation takes place either before or after the potato growth period, the majority of the nematode population will be present in the form of cysts (Evans and Stone, 1977).

Previous studies that evaluated biofumigation effects focus on exposure of juveniles, but not encysted *Globodera* spp., to glucosinolates and their hydrolysed products (Buskov *et al.*, 2002 & Lazzeri, 1993 & Serra *et al.*, 2002 & Warnke *et al.*, 2008). The choice for juveniles instead of cysts seems mainly based on methodological considerations, rather than representing the field situation (Motisi *et al.*, 2010). Only a few studies have used encysted eggs for toxicity tests. The viability of encysted *G. pallida* juveniles was assessed by quantifying nematode actin-1 mRNA in a RT-qPCR assay (Lord *et al.*, 2011). However, this molecular technique does not determine whether the unhatched juveniles would be able to hatch. Juveniles inside the cysts that cannot hatch are of little agronomical relevance (Kroese *et al.*, 2011). Hatching was taken into account by Valdes

*et al.* (2012a), however, no significant effect was observed on encysted *G. rostochiensis* after biofumigation with *Sinapis alba*. Glucosinolate content of *S. alba* was not measured, which might have been too low to cause any effect on hatching of *G. rostochiensis* (Valdes *et al.*, 2012a).

Glucosinolate levels are determined by the interplay of many factors, including plant phenological stage (Rangkadilok *et al.*, 2002), climatic factors (Josefsson and Appelqvist, 1968), insect herbivory and other forms of wounding (Bodnaryk, 1992), availability of nitrogen (N) and S (Gerandás *et al.*, 2009) and plant genotype (Josefsson and Appelqvist, 1968 & Kirkegaard and Sarwar, 1998). Contrasting findings have been reported: sinigrin concentration increased in *Brassica juncea* after herbivory by larvae of the generalist *Spodoptera* spp. (Mathur *et al.*, 2011). However, a significant decrease was found in both total glucosinolates and in sinigrin concentration after feeding by the specialist larvae of *Athalia rosae* (Müller and Sieling, 2006). Besides different insect species being used, Müller and Sieling (2006) used unfertilized soil, while Mathur *et al.* (2011) applied Hoagland solution in their experiment. This suggests that the direction of change for glucosinolate concentrations could be dependent on the insect species, but also on environmental conditions such as nutrient availability.

In this study, we tested the toxicity of 2-propenyl (allyl) isothiocyanate on encysted *G. pallida in vitro*. allyl isothiocyanate is a hydrolysed product of sinigrin, the main glucosinolate of *B. juncea* (Sang *et al.*, 1984). As far as we know, we are the first to report on hatching of encysted *G. pallida* after *in vitro* exposure, in aqueous and vapour phase, to allyl isothiocyanate. Furthermore, we studied *in vivo* the effects of S fertilization and herbivory with *Pieris brassicae* caterpillars on the sinigrin concentration of two *B. juncea* genotypes. *G. pallida* cysts were added to the pots before sowing, and after two months of growth *B. juncea* material was incorporated into its own soil (i.e., biofumigation). We hypothesized that increasing S availability and controlled insect herbivory would increase the sinigrin concentration in *B. juncea*. Increased sinigrin concentration in the plant material was expected to reduce hatching of *G. pallida* through biofumigation. These experiments enabled us to assess whether the sinigrin concentration of *B. juncea* can be manipulated sufficiently to reduce hatching of encysted *G. pallida*.

## 4.2 Materials and Methods

### Cyst population and hatching assessment

The *Globodera pallida* population selected was Pa3 Rookmaker. Cysts were obtained from a lab population from Plant Research International (Wageningen, the Netherlands), and cysts were 2 years old. The Pa3 Rookmaker population is reported to be one of the most virulent populations in the Netherlands and is often used in research and breeding programs (Rouppe van der Voort *et al.*, 1998).

The hatching agent used in this study consisted of root exudates of a mix of five (commercially available) potato cultivars grown in a pot with peat soil in a greenhouse. The pots were watered with excess water once per week, and the leachate was collected. The leachate was filtered through a coffee filter and stored at 7°C. Before use, the leachate was filtered over consecutive sieves of 53, 20 and 5 µm to reduce microbial contamination.

Intact cysts were exposed to allyl isothiocyanate in the toxicity experiment and to compounds released through biofumigation of *B. juncea* (see below). After exposure, intact cysts were extracted and subsequently cleaned and soaked in water for 48 h. After that, cysts were carefully crushed in water using a plunger to liberate the individual eggs and the egg suspension was sieved (150 µm) to remove the cyst walls. The density of the egg suspension (eggs $ml^{-1}$) was determined by counting at least two independent subsamples. Egg suspensions were deposited on sieves (25 µm mesh) that were placed in glass cups containing 1.5 ml hatching agent (undiluted). Mesh size allowed the passage of active *G. pallida* juveniles, while retaining eggs on the sieve. The sieves were occasionally transferred to new glass cups with fresh hatching agent, to count the number of juveniles that had passed the sieve (Been and Schomaker, 2001). Hatching of the nematodes was determined after 7, 13, 20, 34, 55 and 105 days of exposure to the hatching agent in the *in vitro* experiment; and after 14 and 28 days in the *in vivo* experiment. Based on the *in vitro* results, there was no need for longer incubation of the cysts *in vivo*. Hatched juveniles were counted at increasing intervals, because hatching declined over time. The percentage of hatching was calculated by dividing the cumulative number of juveniles that had hatched from a single sieve by the estimated number of eggs. Both dead and living juveniles were counted as hatched. The number of eggs was calculated from the density of eggs in the suspension and the volume of egg suspension (ml) that had been applied to the sieve.

## *Toxicity experiment*

Batches of approximately 200 *G. pallida* cysts (200 eggs per cyst) were prepared by weight (5.3 mg) and exposed to allyl isothiocyanate, either in solution, or in headspace. There were six replicates per combination of exposure time and exposure mode, and batches of cysts were assigned randomly. Exposure times to allyl isothiocyanate were 1, 2, 4, 8, 24 or 168 h, or 0 h in case of the control. Exposure mode was either by mixing cysts into solution (100 ml) in 500 ml bottles, or in headspace by placing the cysts inside a flat bottomed glass vial (14 ml) which was put inside the 500 ml bottle. We dissolved 0.2% allyl isothiocyanate (97% pure, Sigma-Aldrich), and 0.2% Tween-20 as emulsifier, in tap water as stock solution. Based on a pilot experiment, a concentration of 0.002% allyl isothiocyanate was used to conduct the toxicity experiment. From that pilot experiment, it appeared that the length of the soaking period of the cysts influenced hatching. To standardize the time that cysts were dry (in headspace) or soaked (in solution), the cysts for all treatments were prepared simultaneously. Bottles of 500 ml were filled with 99 ml water, and batches of cysts were put either directly into the water, or into a glass vial which was carefully placed in the water. For the longest exposure time treatment (168 h), 1 ml stock solution was immediately added and gently mixed. The shorter exposure-time treatments were initially exposed to water (vapour) and the allyl isothiocyanate was added later. The deviation from the intended exposure time was less than 5 minutes and the cysts in the control were exposed to water only, in solution and in headspace, for the entire week. Cysts were removed from the bottles, washed to remove traces of 2-propenyl isothiocyanate, and dried, after which hatching was determined as described before.

## *Pot experiment*

Sandy soil was collected from an arable field in the vicinity of Wageningen. The soil was selected for its low S levels. Total S was 150 mg $kg^{-1}$ (near-infrared spectroscopy), mineral S was 10 kg $ha^{-1}$ (0-25 cm depth) and the organic matter content was 2.5%. Pots (diameter 20 cm; height 22 cm) were filled with 7 kg of field moist soil (60% water holding capacity). Before filling the pots, about 400 cysts per pot were mixed through the soil for each pot.

We selected two *B. juncea* genotypes: ISCI 99 (labelled “ISCI” in this study, Research Institute for Industrial Crops in Bologna, Italy) was selected for its reported high sinigrin concentration; and IPK CR 2451 (labelled “IPK”, Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany), reported (accession number 485) to be low in sinigrin concentration (Olivier *et al.*,

1999). Three plants were grown per pot, which is comparable to field densities at 27 plants $m^{-2}$ (Larik and Hussain, 1990 & Pradhan *et al.*, 1993). The pots were watered daily based on weight loss. Weeds were removed as soon as they were noticed. There were no traces of unintentional insect infestation. The pots were organized randomly in three blocks and pots were re-randomized weekly within each block (a randomized block design).

Three levels of S availability were obtained using different amounts of Sulfan (12% $NO_3$-N, 12% $NH_3$-N and 15% $SO_3$; Table 4.1). To correct for the N content of Sulfan and to minimize differences in salt level and pH between the fertilizer treatments, calcium ammonium nitrate ($CaNH_4NO_3$; 13.5% $NO_3$-N, 13.5% $NH_3$-N and 6% $CaCO_3$) was applied. Fertilizer was applied in two steps; the first dose of 1 g per pot was given 7 days after emergence, followed by a second dose of 1 g per pot 10 days later.

*Table 4.1.*

Fertilizer application rates (kg $ha^{-1}$) with calcium ammonium nitrate (CAN) and Sulfan, and total nitrogen (N) and sulphur (S), applied in the greenhouse pot experiment

| S level | Fertilizer type | | Nutrient amount | |
|---|---|---|---|---|
| | CAN | Sulfan | N | S |
| Low | | 0 | 48.6 | 0.0 |
| Medium | 90 | 90 | 45.9 | 13.5 |
| High | 0 | 180 | 43.2 | 27.0 |

Caterpillar eggs of *P. brassicae* (Laboratory of Entomology, Wageningen UR) were introduced one month after emergence of the *B. juncea* seedlings, placing three eggs per plant. Incomplete hatching of the caterpillars occurred. Therefore, the number of caterpillars was equalized by transferring caterpillars to obtain three to four individuals per pot. When the majority of caterpillars reached the stage of fifth instar, caterpillars were removed from the plants to avoid pupation and complete consumption of the plants. The pots with caterpillars were placed in a split plot design within each block for the duration of feeding (2 weeks), to minimize the risk of unintentional caterpillar damage on plants without caterpillars. Occasional migration of caterpillars within the split plots could have occurred.

After 2 months of growth, we selected three almost full-grown leaves from the middle of the plant for glucosinolate analysis. These specific leaves were selected to minimize variation caused by phenological differences of the genotypes. At sampling, IPK was at the transition to the generative phase, while ISCI was already setting seed. Leaf material was freeze-dried, ground with a ball mill and analysed for glucosinolates by HPLC and mid IR spectroscopy (Schütze *et al.*, 2004).

The shoot material that remained after taking samples for glucosinolate quantification was chopped up manually with pruning shears, chopped-up shoot parts were immediately mixed into the soil. The soil, including the root parts, was split in two parts: 4 kg was mixed with 50 g fresh weight of the chopped-up shoot parts (realistic biofumigation amounts in field conditions) and returned to the pot for the biofumigation treatment. The pots were subsequently watered and individually covered with a plastic bag (but not completely sealed) and kept in the greenhouse for 6 weeks. The remaining soil from each pot, and the cysts herein, was stored in plastic bags at 4 °C until extraction of cysts (4 weeks) for determination of natural decline in hatching during the growth period only. The shoot material that remained after taking 50 g for biofumigation was dried to determine dry weight concentration for conversion of glucosinolate concentration in the plant to the amount of glucosinolates per pot.

A scaled-up Seinhorst elutriator was used to retrieve the cysts (plus other organic matter) from the biofumigated and the non-fumigated soil (Been *et al.*, 2007). All samples were air-dried, followed by sieving and manual collection of cysts from the remaining debris. Hatching was determined using the same protocols as described before. The number of hatched juveniles was determined after 14 and 28 days of exposure to the hatching agent (potato leachate).

The mean temperature during the growth period of the plants was 19°C. The daily mean temperature fluctuated between 17.3 and 21.7°C. Minimum and maximum temperatures recorded were 13.0 and 25.6°C, respectively. During the biofumigation period, the mean inside temperature was 18.1°C with extremes of 11.9 and 23.0°C. Relative humidity (RH) during the growing period was on average 59%. During the following biofumigation period, the average RH was lower: 45%, with extremes of 21 and 73%. Supplemental light was provided during the growth period to provide 16 h of light per day.

### *Data analyses*

We determined the cumulative percentage of hatching after 105 days in the *in vitro* toxicity experiment. We tested the effects of exposure time, exposure mode and their interaction on the percentage of hatching using 2-way ANOVA (Genstat 15$^{th}$ Edition SP1). Least significant difference was used to identify differences between treatments. Furthermore we determined the median lethal time ($LT_{50}$) for encysted eggs for a concentration of 0.002% allyl isothiocyanate. For the *in vivo* experiment the cumulative percentage of hatching was determined after 28 days. We tested the effects of genotype, S availability, and feeding of *P. brassicae*, and their interactions, on the sinigrin concentration of the plants, on the percentages of hatching after plant growth, and after biofumigation by using 3-way ANOVA. We used Pearson's correlation coefficient to investigate correlations between sinigrin concentration and the percentage of hatching both after the growing period and at the end of the incubation period. All residuals were distributed normally and variance was homogenous based on the Kolmogorov-Smirnov test.

## *4.3 Results*

### *Toxicity experiment*

We observed a negative effect of exposure time ($F = 6.95$, $p < 0.001$) on *G. pallida* hatching exposed to 0.002% allyl isothiocyanate, but no effect of exposure mode (headspace or in solution). Hatching was on average 74% for the 0-hour treatment which served as control, and decreased with increasing exposure time (Fig 4.1a). Interpolation ($y = 0.69 \cdot x^{-0.69}$, $R^2 = 0.99$) resulted in a median lethal time ($LT_{50}$) of 1.85 h for hatching of *G. pallida* after exposure to 0.002% allyl isothiocyanate.

### *Pot experiment*

The sinigrin concentration of *B. juncea* was affected by the interaction between genotype, S level, and caterpillar feeding (*P. brassicae*) (Table 4.2). Without *P. brassicae* herbivory, sinigrin concentration increased with increasing S levels for both genotypes (Fig. 4.1b). With *P. brassicae* present, only genotype ISCI further increased in sinigrin concentration at the highest S fertilization. Genotype IPK yielded a high sinigrin concentration at medium S fertilization with *P. brassicae* herbivory. The only treatment in which genotype ISCI yielded a higher sinigrin concentration was at high S fertilization with insect feeding.

*Table 4.2.*

Effects of *B. juncea* genotype (IPK or ISCI), sulphur availability (low, medium or high) and insect feeding (absent or present), and their interactions, on sinigrin concentration and hatching percentage after crop growth, or after crop growth and additional biofumigation

| | Sinigrin concentration | | Hatching percentage | | | |
|---|---|---|---|---|---|---|
| | | | After crop growth | | After crop growth and biofumigation | |
| | *F* | *p* | *F* | *p* | *F* | *p* |
| Genotype (G) | 6.01 | **0.02** | 0.74 | 0.39 | 0.03 | 0.86 |
| Sulphur (S) | 39.17 | **<0.001** | 1.02 | 0.37 | 2.31 | 0.12 |
| Feeding *P. brassicae* (F) | 6.74 | **0.01** | 0.30 | 0.59 | 0.49 | 0.49 |
| G * S | 4.79 | **0.01** | 2.03 | 0.15 | 0.84 | 0.44 |
| G * F | 0.02 | 0.88 | 3.79 | 0.06 | 0.00 | 0.98 |
| S * F | 1.33 | 0.28 | 0.98 | 0.38 | 0.07 | 0.93 |
| G * S * F | 3.43 | **0.04** | 2.41 | 0.10 | 0.75 | 0.48 |

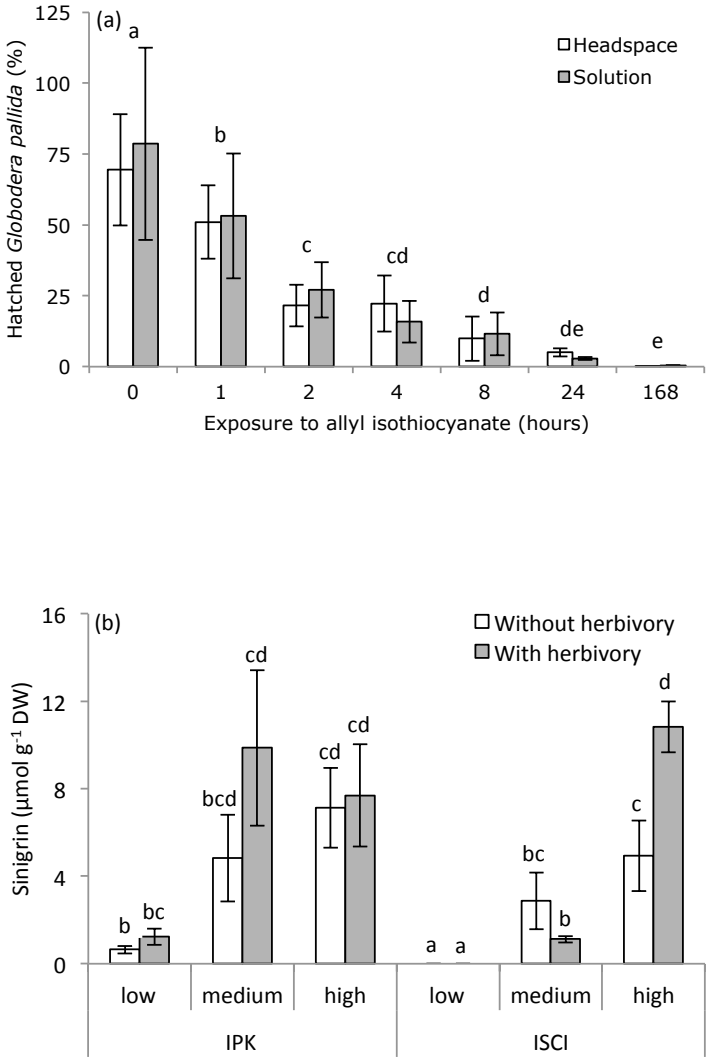


*Figure 4.1.*

Percentage (a) of hatched *Globodera pallida* for 2 exposure modes: headspace (gaseous) and solution (liquid), for the control (0 h) and after exposure to 0.002% allyl isothiocyanate. Different letters indicate statistically significant differences between exposure times (LSD, P<0.05) and error bars are standard errors of the mean (n = 3). Concentration of sinigrin (b) in two *Brassica juncea* genotypes (IPK and ISCI), with 3 sulphur fertilization levels (low, medium and high) with and without *Pieris brassicae* herbivory. Different letters indicate statistically significant differences between treatment combinations (LSD, P<0.05) and error bars are standard errors of the mean with n = 3 (n = 12 for the first and last bar in the graph).

None of the treatments differentially affected the hatching of *G. pallida*, neither before nor after biofumigation (Table 4.2). We did not observe any correlation between shoot sinigrin concentration and juvenile hatching from cysts that were exposed only to crop growth ($r$ = 0.19, $p$ = 0.17), or from cysts that were additionally exposed to biofumigation ($r$ = -0.07, $p$ = 0.59). We did find a decline in hatching but this was independent of the experimental factors (Table 4.3). Initial juvenile hatching from the cysts was 70% (SE ±2). Hatching was reduced to 39% (SE ±2) after two months of crop growth, before biofumigation. Hatching was further reduced to 26% (SE ±1) after incorporation of *B. juncea* tissue and a 6 week incubation period, i.e. biofumigation.

*Table 4.3.*

Average hatching percentage of encysted *Globodera pallida* juveniles for each *Brassica juncea* genotype, sulphur level, and feeding of *Pieris brassicae* (or without). Hatching is presented after crop growth, and after crop growth and biofumigation, standard errors (±), n = 3 for all, except n = 12 for genotype IPK with low sulphur without herbivory (first line of table), and for genotype ISCI with high sulphur with herbivory (last line of the table).

| Genotype | Sulphur | Herbivory[a] | After crop growth | After crop growth and biofumigation |
|---|---|---|---|---|
| IPK | Low | - | 0.39 ±0.04 | 0.26 ±0.03 |
| | | + | 0.45 ±0.08 | 0.23 ±0.02 |
| | Medium | - | 0.20 ±0.03 | 0.32 ±0.05 |
| | | + | 0.43 ±0.02 | 0.30 ±0.02 |
| | High | - | 0.38 ±0.09 | 0.21 ±0.04 |
| | | + | 0.27 ±0.06 | 0.28 ±0.03 |
| ISCI | Low | - | 0.44 ±0.09 | 0.27 ±0.12 |
| | | + | 0.26 ±0.04 | 0.26 ±0.07 |
| | Medium | - | 0.41 ±0.12 | 0.25 ±0.05 |
| | | + | 0.31 ±0.09 | 0.33 ±0.14 |
| | High | - | 0.46 ±0.07 | 0.20 ±0.05 |
| | | + | 0.42 ±0.04 | 0.22 ±0.02 |

[a] with (+) or without (-) *Pieris brassicae* caterpillars

## 4.4 Discussion

We observed a high toxicity of allyl isothiocyanate on encysted *G. pallida* eggs *in vitro*. Hatching was reduced by 50% compared to the control after less than 2 h of exposure to 0.002% allyl isothiocyanate in solution as well as in headspace (Fig. 4.1a). It is obvious that allyl isothiocyanate was able to pass into the cyst and affect the juveniles inside, both in liquid and gas phase. How allyl isothiocyanate affects *G. pallida* juveniles inside the cysts and inhibits hatching is unknown. In *Caenorhabditis elegans*, allyl isothiocyanate stimulated the expression of heat shock proteins, which are involved in the response to stress (Saini *et al.*, 2011). The expression of heat shock proteins has generally a high energy cost (Mitra and Bhatia, 2008). Excessive energy that is used for expression of heat shock proteins could be the cause of the inability to hatch of the encysted juveniles. In our experiment some juveniles died after hatching, which means that they were able to pass the sieve or were released during crushing of the cysts, but were not able to survive in the leachate. This could be a consequence of starvation due to excess energy costs of heat shock proteins (Storey, 1984). Understanding the underlying mechanisms is necessary to improve the focus of future research into the sensitivity of potato cyst nematodes and other organisms to allyl isothiocyanate.

It was found that sinigrin, the precursor of allyl isothiocyanate, increased in *B. juncea* with *P. brassicae* herbivory and fertilization (Fig. 4.1b). In our experiment, we selected genotype ISCI for its claimed high levels of sinigrin, but we did not observe higher sinigrin concentrations in ISCI than in genotype IPK. Levels of sinigrin are known to change with phenological stage and also differ between tissue types. Seed pod development results in a drop of sinigrin levels in the stems and leaves, while the sinigrin levels in the seeds and seedpods have been reported to be two to twelve times higher than the rest of the shoot (Rangkadilok *et al.*, 2002). It is possible that the sinigrin values we report are an underestimation for the entire shoot, because we measured the sinigrin levels in leaves when the seed pods were already developing in ISCI (but not in IPK). This could explain why sinigrin concentrations are below the detection threshold in the leaves of genotype ISCI at low S fertilization.

Feeding of *P. brassicae* only increased the sinigrin concentration significantly in genotype ISCI at high S fertilization (Fig. 4.1b). The range of sinigrin concentrations observed in our study was in the same order of magnitude as those reported by Mathur *et al.* (2011) and Vervoort *et al.* (2014). However, larger differences between *B. juncea* plants with and without herbivory were observed by Mathur *et al.* (2011). In genotype ISCI the sinigrin concentration increased from medium to high S fertilization, but we could not detect sinigrin at low S fertilization (Fig. 4.1b). Gerendás *et*

*al.* (2009) observed a lack of response to higher S fertilization when N availability became the new plant growth-limiting factor. In our experiment, it is also possible that another nutrient became limiting after the level of S had been raised sufficiently. Sulphur uptake was increased by selenium fertilization in *Brassica oleracea*, however, this resulted in a decreased sinigrin concentration (Toler *et al.*, 2007). This suggests that S interacts with other nutrients in sinigrin metabolism. Untangling the effect of environmental constraints on sinigrin metabolism could result in improved fertilizer recommendations with the potential to increase sinigrin concentration.

Higher sinigrin concentrations of incorporated *B. juncea* tissues did not result in an decrease in hatching of *G. pallida*. For biofumigation, we chopped and incorporated 50 g fresh weight *B. juncea* shoots into 4 kg of soil. The highest measured sinigrin concentration of the added material was 16 µmol $g^{-1}$ shoot dry weight. Dry matter concentration of the shoots was on average 20%, resulting in an estimated amount of 160 µmol sinigrin in 4 kg of soil. Assuming all sinigrin to be hydrolysed into allyl isothiocyanate instantaneously, 160 µmol allyl isothiocyanate would have been released. With an average soil water level of 20% this equals 200 µmol allyl isothiocyanate $l^{-1}$ soil water or, based on the molar weight, 0.002% equal to the highly toxic concentration used in our *in vitro* assay. However, a release efficiency of glucosinolate into isothiocyanate of 1-26% is probably more realistic (Morra and Kirkegaard, 2002), so the concentration in the pots was probably lower than 0.002%. Low effectiveness of isothiocyanates could be caused by organic matter content and pH of the soil used (e.g. Borek *et al.*, 1995 & Matthiessen and Kirkegaard, 2006). From the lack of reduction in hatching following biofumigation, we must conclude that we were not able to obtain lethal allyl isothiocyanate concentrations in our pots. Our calculations suggest that biomass from at least 100 ha of *B. juncea* would be needed to control *G. pallida* infestation in 1 ha. To increase the sinigrin concentration in the soil to sufficient levels, different strategies can be used. The sinigrin concentration in the plant material could, theoretically, be further increased, or the ratio of plant material to soil could be increased. The ratio we used in our pot experiment, realistic for field conditions, corresponds to the incorporation of a total aboveground biomass of 25 Mg $ha^{-1}$ to a 10 - 12.5 cm depth in the field. This ratio could be increased by shallower incorporation, thereby concentrating the plant material, but this would likely leave cysts in deeper layers unexposed.

## *4.5 Conclusions*

The results of these experiments demonstrate a toxic effect of isothiocyanates on potato cyst nematodes *in vitro*. In addition, we show significant effects of fertilization, insect herbivory and genotype on plant sinigrin concentration. However, after biofumigation we did not observe any effect of increasing plant tissue glucosinolate concentrations on the hatching of the potato cyst nematodes. Although it proved possible to manipulate glucosinolate concentrations in *B. juncea*, we were not able to increase allyl isothiocyanate concentrations to levels sufficient to cause toxic effects on encysted *G. pallida* in our, agronomically relevant pot experiment.

# Chapter 5

## Effects of GM potato cultivar Modena on soil biota remain within the range of effects induced by genotypic variation

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## *Abstract*

As soils constitute complex habitats with a high biodiversity and often in close proximity to roots, alterations in plant composition induced by genetic modification (GM) is expected to influence soil processes. Here, we investigated the impact on soil microbial activity of a marker-free GM potato (*Solanum tuberosum* cv. Modena) blocked in its amylose biosynthesis pathway. A field experiment was conducted at two sites in which microbial catabolic diversity was assessed at two time points in plots grown with the GM cultivar Modena, its parental isoline Karnico and the conventional cultivar Aventra. In addition, we assessed whether plant litter decomposing under controlled conditions resulted in distinct effects on carbon and nitrogen cycling and if Modena prompted a home field advantage, i.e. plant litter might decompose more rapidly in soil from which it was derived from than from away soil. No GM induced changes were observed in microbial activities, whereas significant effects of location and time of sampling were detected. Analysis with plant traits and soil characteristics revealed that soil moisture explained a large part of the variation in microbial catabolic diversity data in bulk soil. In the rhizosphere, soil organic matter and tuber sucrose content affected microbial catabolic diversity. The only observed GM induced effect was a slight home field advantage for Modena in nitrogen mineralization, yet this was not consistent at both locations. In combination with results from previous studies on other components of the soil food web, we conclude that the GM cultivar Modena had no immediate effects on soil microbial activity as compared to conventional potato cultivars.

## 5.1 Introduction

Since the commercialization of genetically modified (GM) crops in 1996, the area of croplands cultivated with GM varieties increased up to 175 million hectares in 2013 (James, 2013 & Privalle *et al.*, 2012). The area of biotech crop is unevenly distributed, about two-third of this area is located in the Americas. In The Netherlands, no GM crops are commercially grown due to concerns about possible side effects on the environment and ecosystem services (e.g. Bruinsma *et al.*, 2003). Depending on the nature of the modification, soil systems could be affected by GM plants through a trait-mediated shift in root physiology, root exudates and/or litter quality (Powell, 2007). Possible effects during the growing season induced by GM crops are therefore most likely to occur in the rhizosphere, a thin zone around the roots and a hotspot for microorganisms (Hartmann *et al.* 2008). However, in terms of risk assessment it is relevant to know whether plant-induced effects in the rhizosphere would have prolonged effects on soil biota (Mulder *et al.*, 2006 & Powell, 2007).

Current techniques allow commercial development of marker-free GM crops, i.e. cultivars without plant-transformation markers such as kanamycin resistance. One of these crops is a potato (*Solanum tuberosum*) cultivar that is modified in starch composition. Normally, starch in potato tubers consists of amylose and amylopectin in a ratio of about 1/5 (Broothaerts *et al.*, 2007), but tubers of the cultivar Modena (BASF GmbH, Limburgerhof, Germany) produce amylose-free starch. This was accomplished by the inhibition of the transcription of the granule-bound starch synthase gene, which is essential for amylose production, in the parental cultivar Karnico. The resulting amylose-free starch is an attractive ingredient for a range of industrial applications (Visser and Jacobsen, 1993). Since this particular trait is a direct inhibition of a pathway in the carbohydrate household and a plant-transformation marker is absent (De Vetten *et al.*, 2003), possible environmental effects due to this GM crop can be related to its metabolism.

Several studies assessed the effects of Modena and similar potato genotypes modified in starch content on soil biota (e.g. Milling *et al.*, 2005 & Hannula *et al.*, 2010). Induced differences by Modena were mainly observed in the rhizosphere and at the senescence growth stage of the plant with the effects assumed to be caused by root exudates (İnceoğlu *et al.*, 2013). In a recent study, Dias *et al.* (2013) observed that the relative proportions of gram-negative bacteria *Pseudomonas* and *Burkholderia* spp. were different in rhizosphere of Modena and Karnico. However, several studies showed that potato age, soil types and yearly fluctuations had more prominent effects on the rhizobiome as compared to potato cultivar induced effects (Gschwendtner *et al.*, 2011 & Hannula *et al.*, 2012a,b & İnceoğlu *et al.*, 2012, 2013).

Next to a possible distinct root exudation pattern, alteration in the carbohydrate household may also affect other plant parts in Modena and could result in an altered litter quality (Jacobsen *et al.*, 1989 & Privalle *et al.*, 2013). Dissimilar litter qualities can affect decomposition processes. Hannula *et al.* (2013) observed faster decomposition rates of Modena tubers and leaves compared to its parental cultivar Karnico after one month, but no differences in rates were observed after 3 and 6 months of incubation. Since root exudation of Modena may result in an altered rhizobiome and the trait can affect litter quality, potato cultivar Modena might induce a home field advantage. The home field advantage concept is that litter decomposes more rapidly in soil from which the plant is derived than a different plant (Ayres *et al.*, 2009).

The first aim of this study was to compare the microbial catabolic diversity in rhizosphere and bulk soil from potato cultivars Modena (GM), Karnico (parental), and Aventra (conventional cultivar) and link observed effects to potato traits. Secondly, we investigated whether Modena prompted a home field advantage in decomposition processes by measuring the effects of plant litter decomposition on soil carbon (C) and nitrogen (N) cycling. The conventional cultivars (Aventra and Karnico) were included in our experiments to frame potential GM trait-related effects in the background of variation due to genotypic variation (Griffiths *et al.*, 2007 & Perry *et al.*, 2009). We hypothesize that effects induced by GM cultivar Modena on soil biota and soil processes are not exceeding the range of effects induced by conventional potato genotypes.

## *5.2 Material and Methods*

### *Experimental design*

The experiment was performed at two experimental fields (VMD and BUI) with the same soil acidity (pH in $H_2O$ 4.6), located at ~10 km of each other in the province of Drenthe, The Netherlands. Soil from VMD was characterised as a sandy peat and soil from BUI as loamy sand. The fields were distinct in soil characteristics, mainly for organic matter, but also for sand fraction and average particle size of the sand fraction (Table 5.1).

*Table 5.1.*

Soil characteristics of the fields at the locations VMD and BUI.

| | VMD | BUI |
|---|---|---|
| pH | 4.6 | 4.6 |
| Organic matter (%) | 25.5 | 5.3 |
| Bulk density (g $cm^{-3}$) | 1.38 | 1.05 |
| Silt fraction (% < 16 µm) | 2.8 | 5.7 |
| Sand fraction (% > 50 µm) | 94.3 | 90.5 |
| Particle size sand fraction (µm) | 157.7 | 130.0 |

At each experimental field the potato (*S. tuberosum*) cultivars Aventra, Karnico, and Modena were grown. Each field consisted of four replicate plots per cultivar (24 plots in total), which were distributed using a randomized complete block design. Cropping in these sites consisted of potato-barley-barley rotation. Potatoes were seeded at April 27th and each plot contained twenty eight plants divided over four ridges. Distance between ridges was 75 cm and 40 cm between individual potato tubers within a ridge. Fertilizer application consisted of standard potato growth applications. At location VMD, potatoes were fertilised with 140 kg N $ha^{-1}$, 50 kg $P_2O_5$ $ha^{-1}$ and 1500 kg protamylase (potassium-rich product) $ha^{-1}$, and at location BUI with 250 kg N $ha^{-1}$, 250 kg $K_2O$ $ha^{-1}$ and additionally 20,000 kg pig slurry $ha^{-1}$.

## *Sampling*

At both locations, soil samples were taken in 2011 at August 15th and September 13th. Per plot, a composite soil sample of the bulk soil was taken, consisting of 16 cores (Ø 2 cm, depth 20 cm) taken from the four potato ridges in each plot. Rhizosphere sampling was done by carefully lifting potato plants including root-adhering soil using a spade. Plants were shaken twice and the soil that was subsequently brushed from the roots was used as rhizosphere soil. All samples were chilled after sampling (4 °C).

For the decomposition experiment, including the home field advantage assessment, we collected plant shoots on September 13th. Potato shoot material was dried (70 °C for 24 h), ground, and analyzed for C and N content using flash combustion in an elemental analyzer (EA 1108) (Pella and Colombo, 1973). Potato tuber starch, sucrose, vitamin C and glycoalkaloids were extracted and analyzed as described in detail by Privalle *et al.* (2013) from additional trials. These trials were located adjacent to the experimental fields in which we assessed microbial activities at both locations.

## *Microbial catabolic diversity*

Measurements of the catabolic activity of the microorganisms was done according to the MicroResp™ procedure (Campbell *et al.*, 2003). Carbon substrates used were alanine, arginine, citric acid, glucose, fumaric acid, malic acid, oxalic acid, phenylalanine, sucrose, trehalose and tween-80 and water as control. Substrates selection was based on plant root exudation pattern (Campbell *et al.*, 1997). Substrates were dissolved in demineralised water and prepared as stock solutions designed to deliver 30 mg C $g^{-1}$ soil solution after dispensing 25 µl of the solution. Based on the solvability, lower concentrations (7.5 mg C $g^{-1}$ soil solution) were used for substrates alanine, arginine, phenylalanine, oxalic acid and fumaric acid. The substrate solution was dispensed into a deepwell plate, and each substrate was dispensed into four replicate wells. Soil samples were sieved (2 mm) and added to the deepwells using the standard filling device to add about 300 µl of soil to each well. Moisture content of the soil samples was determined (drying at 105 °C for 24 h) and fresh soil weight added to the deepwell was determined and used to calculate the average weight of soil per well for each sample. Absorbance of the indicator dye in the detection plate was measured at 570 nm before and after the incubation period (25 °C for 6 h) using a micro plate reader (VMAX, Molecular Devices). Colour development was normalized by subtracting the averaged time zero measurements for each plate from the measured colour development per well after 6 h incubation according to Campbell *et al.* (2003) and converted to $CO_2$ concentrations using a calibration curve; $\%CO_2 = 0.002 \cdot A_{570}^{-3.11}$, $R^2 = 0.93$. Where $\%CO_2$ (v/v) is the concentration in the headspace after incubation and $A_{570}$ is the normalized absorbance. Median $CO_2$ concentrations ($n = 4$) for each substrate per plot were converted to respiration rates (µg $CO_2$-C $g^{-1}$ soil $hr^{-1}$) using soil fresh weight and soil water content. Median respiration rates of the control, *i.e.* water, were subtracted from the median respiration rates induced by a C source.

## *Decomposition processes (home field advantage)*

From each experimental plot, four bottles (333 ml) were filled with 75 g soil (fresh weight). Moisture content of the soil was gravimetrically adjusted to 60% of the water holding capacity throughout the experiment. Three out of the four bottles received additional 1.5 g potato shoot material (dry weight) from the cultivar (Karnico, Modena and Aventra) sampled from the same experimental block, and plant material was mixed into the soil. The litter induced decomposition rates were determined in the so called 'home situation' (plant material from cultivar grown on same plot, bottle one) and in the 'away situation' (plant material from the other cultivars, bottle two and three). The fourth bottle received no additional shoot material, and was used to determine basal decomposition rate.

Microbial biomass N was determined in all samples following the chloroform fumigation and extraction technique, using 0.5 M $K_2SO_4$ as extractant (1/4 w/vol, fresh weight basis) ($k_{EC}$ = 0.54) (Brookes *et al.*, 1985). Total dissolved N from the non-fumigated samples was used to determine net N mineralization. Nitrogen content was measured at day 0 and at the end of the incubation period (day 121) using a segmented flow analyser (Skalar Analytical B.V. Breda, The Netherlands). The bottle without additional plant material (bottle four) was subtracted from the potato shoot induced values for microbial biomass N and for net N mineralization. The change in microbial biomass N was calculated over the incubation period (µg N $g^{-1}$ dry soil), net N mineralization is expressed per day (µg N $g^{-1}$ dry soil $day^{-1}$).

Carbon mineralization was determined in all samples over the incubation period of 121 days. $CO_2$ production was measured after 14, 17, 23, 37, 65 and 121 days of incubation. During sampling, each bottle was flushed with compressed air for 15 min closed with an air tight lid and incubated for 4 hrs at 20 °C in the dark, after which the $CO_2$ concentration was measured with a photo-acoustic field gas-monitor (INNOVA 1412, Lumasense™ Technologies). Every week, soil moisture content was adjusted gravimetrically. Basal C mineralization (fourth bottle) was subtracted from the potato shoot induced mineralization. For each cultivar per experimental plot, the area under the curve of C mineralization over time was calculated for the incubation period and mineralization rates were expressed as µg $CO_2$ $g^{-1}$ dry soil $day^{-1}$.

Home field advantage was calculated for C mineralization, microbial biomass N content and N mineralization based on the equations used by Ayres *et al.* (2009):

$$ADH_i = HDD_i - ADD_i - H$$

$$HDD_i = (D_{ii} - D_{ji}) + (D_{il} - D_{kl})$$

$$ADD_i = (D_{ii} - D_{ji}) + (D_{iK} - D_{kK})$$

$$H = (HDD_i + HDD_j + HDD_k) / (N - 1)$$

Where ADH is the additional decomposition at home for cultivar *i*; *i*, *j* and *k* are potato shoot materials derived from the three potato cultivars Karnico, Modena and Aventra respectively; *I*, *J* and *K* are soil samples from the plots where cultivars *i*, *j* and *k* were grown, respectively. HDD and ADD represent home decomposition difference and away decomposition difference, respectively; *H* represents the total home field advantage for all species combined; and *N* represents the number

of cultivars. A home field advantage is present when $ADH_i > 0$, for example if C mineralization of potato plant material from Modena on soil derived from Modena plots was higher than expected. $ADH_i = 0$ indicates that mineralization was similar as expected (no home field advantage and no home field disadvantage), while $ADH_i < 0$ occurs when mineralization of potato material at home was lower than expected (a home field disadvantage).

## *Data analyses*

The effects of location, time of sampling and cultivar on the microbial catabolic diversity was assessed in rhizosphere and bulk soil using the permutation tests by redundancy analysis (RDA) (CANOCO; version 5.0) (Van Dobben *et al.*, 1999). Monte Carlo permutation tests (999 random permutations) were performed to indicate effects on the dependent variables. Since effects of location and time of sampling were detected, we included soil moisture, soil organic matter, and potato tuber glycoalkaloids, starch, sucrose and vitamin C content in a follow-up analysis. These tuber components comprised distinct effects among the cultivars (Privalle *et al.*, 2013). In the RDA analysis, forward selection, i.e. stepwise selection of explanatory variables, was used to select for variables comprising the strongest effects and sort-out variance that already was explained by a former variable. Block effects were partialized out by entering block indicator variables as covariables. Explanatory variables that comprised significant associations ($p < 0.05$) to the microbial activities were included in ordination plots.

For the decomposition processes, ANOVA was used to analyse the effects of cultivar, location and the interaction between cultivar and location at the home situation. For these processes, the area under the curve over the incubation period for C mineralization, the increase in microbial biomass N, and the N mineralization rate were tested for both basal and potato induced decomposition. In addition, cultivar and location effects were tested on the C/N ratio of potato shoot material.

For the home field advantage assessment, we calculated the average and 95% confidence interval (average ± 1.96 × SE) for the additional decomposition at home (ADH) with the assumption of a normal distribution of the data. If the 95% confidence interval did not include zero, we considered this as a home field advantage (ADH > 0) or a home field disadvantage (ADH < 0).

## 5.3 Results

### *Microbial catabolic activities in two soil zones*

No cultivar effects were observed on the microbial catabolic diversities in rhizosphere or in bulk soil. Main effects were observed for location (67.3% explained variance, $F = 85.2$, $p < 0.002$, based on RDA) and time of sampling (4.8% explained variance, $F = 3.1$, $p = 0.02$) on microbial catabolic diversities in the rhizosphere. Similarly, bulk soil microbial catabolic diversities were affected by location (53.3% explained variance, $F = 47.8$, $p < 0.002$) and by time of sampling (16.1% explained variance, $F = 8.9$, $p < 0.002$).

The follow up analysis, including plant traits and soil characteristics as explanatory variables, revealed that soil moisture affected the microbial catabolic activities in bulk soil, whereas organic matter and tuber sucrose content affected microbial catabolic activities in the rhizosphere (Table 5.2). In the rhizosphere, microbial activities upon addition of sucrose, arginine, tween 80, trehalose, phenylalanine, and malic acid were positively associated with organic matter content (Fig. 5.1a).

*Table 5.2.*

Overview of the effects of explanatory variables derived from potato tuber content and soil characteristics, expressed as variance explained, pseudo-*F* and *p*-values, on the microbial catabolic diversities in rhizosphere and bulk soil of the three potato cultivars (Aventra, Karnico and Modena). Based on redundancy analysis this shows that potato tubers content, glycoalkaloid (mg $kg^{-1}$ fresh weight), starch (g 100 $g^{-1}$ fresh weight), sucrose (g 100 $g^{-1}$ fresh weight) and vitamin C (mg 100 $g^{-1}$ fresh weight), had no or small effect (explained variance <2.5%), while soil organic matter content (%) and soil moisture (g water 100 $g^{-1}$ dry soil) strongly influenced on microbial catabolic diversity in the rhizosphere and in the bulk soil, respectively. Significant explanatory variables in bold.

| | | Rhizosphere | | | Bulk | | |
|---|---|---|---|---|---|---|---|
| | | Var (%) | *F* | *p* | Var (%) | *F* | *p* |
| Potato | Glycoalkaloids | 0.6 | 0.8 | 0.49 | 1.2 | 1.0 | 0.31 |
| | Starch | 1.6 | 2.2 | 0.09 | 0.3 | 0.2 | 0.95 |
| | Sucrose | 2.4 | 3.1 | **0.03** | 0.2 | 0.2 | 0.94 |
| | Vitamin C | 1.0 | 1.2 | 0.27 | 0.4 | 0.3 | 0.85 |
| Soil | Organic matter | 65.1 | 80.2 | **<0.002** | 0.2 | 0.2 | 0.97 |
| | Moisture | 0.5 | 0.6 | 0.72 | 48.8 | 41.0 | **<0.002** |

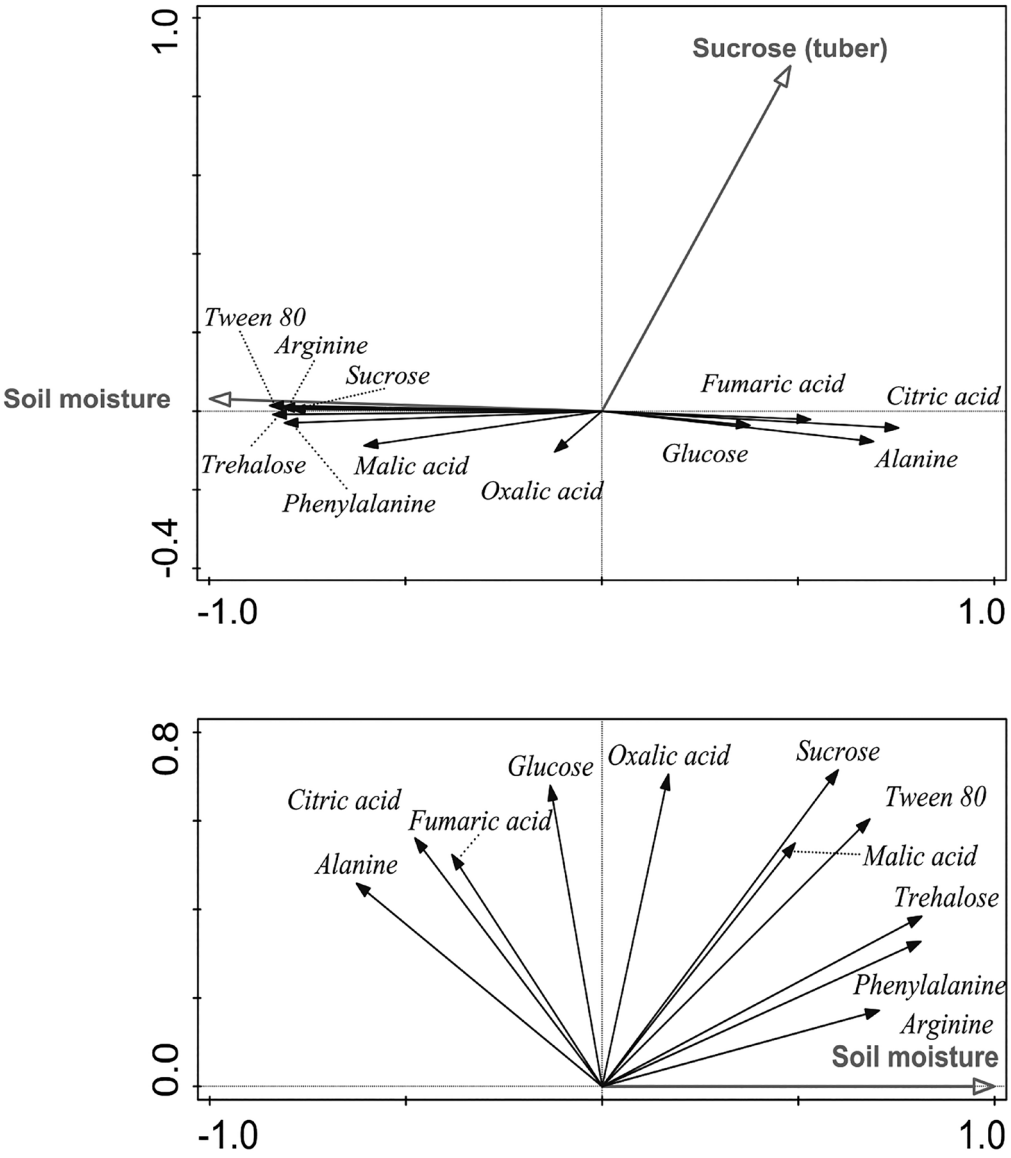


*Figure 5.1.*

Ordination diagram of the microbial catabolic diversities in rhizosphere (a) and in bulk soil (b) of the potato cultivars based on RDA. Tuber sucrose content and soil organic matter are included as explanatory variables in the microbial catabolic diversity in the rhizosphere (accounting for 63.6% of the variance) and soil moisture is included for the bulk soil (accounting for 43.4% of the variance).

Negative associations were observed between soil organic matter and induced microbial respiration upon the addition of fumaric acid, citric acid, alanine and glucose. Sucrose content of the potato tubers was negatively associated with induced microbial respiration upon the addition of arginine, phenylalanine, trehalose, tween-80, malic acid, sucrose and oxalic acid. In the catabolic response of the microbial community in bulk soil, moisture content was positively associated with induced microbial respiration upon the addition of arginine, phenylalanine, trehalose, tween-80, malic acid and sucrose (Fig. 5.1b). Negative associations were observed between soil moisture and induced microbial respiration upon the addition of fumaric acid, citric acid, and alanine.

## *Decomposition processes*

Litter material used in the decomposition experiment had a comparable quality, as indicated by the C /N ratio of the potato shoots which was not affected by cultivar ($F$ = 3.30, $p$ = 0.07) or location ($F$ = 2.98, $p$ = 0.11) (Fig. 5.2a). Potato cultivar Modena grown at location VMD and cultivar Karnico grown at location BUI showed a home field advantage for net N mineralization (Table 5.3). A home field disadvantage was observed for cultivar Aventra at both locations for net N mineralization.

*Table 5.3.*

Estimation of home field advantage (ADH; average and 95% confidence interval) in litter decomposition, expressed for carbon mineralization, nitrogen mineralization and microbial biomass nitrogen for the three potato cultivars (Karnico, Modena and Aventra) at both locations (VMD and BUI). Significant results in bold ($n$ = 4).

| | | C mineralization ADH | | N mineralization ADH | | Microbial biomass N ADH | |
|---|---|---|---|---|---|---|---|
| | | average | 95% CI | average | 95% CI | average | 95% CI |
| VMD | Karnico | 0.78 | (-4.20 – 5.76) | 0.62 | (-0.29 – 1.53) | 0.21 | (-0.62 – 1.03) |
| | Modena | 1.84 | (-1.33 – 5.01) | **1.02**[a] | **(0.20 – 1.84)** | -0.13 | (-0.85 – 0.60) |
| | Aventra | -2.01 | (-5.24 – 1.23) | **-1.61**[b] | **(-3.03 – -0.19)** | -0.04 | (-0.74 – 0.66) |
| BUI | Karnico | -0.77 | (-4.17 – 2.62) | **0.62**[a] | **(0.08 – 1.15)** | -0.29 | (-0.69 – 0.12) |
| | Modena | 0.10 | (-1.92 – 2.13) | 0.26 | (-0.55 – 1.07) | -0.07 | (-1.40 – 1.26) |
| | Aventra | 1.21 | (-2.38 – 4.80) | **-0.90**[b] | **(-1.66 – -0.15)** | 0.35 | (-0.67 – 1.37) |

[a] Home field advantage (ADH>0)
[b] Home field disadvantage (ADH<0)

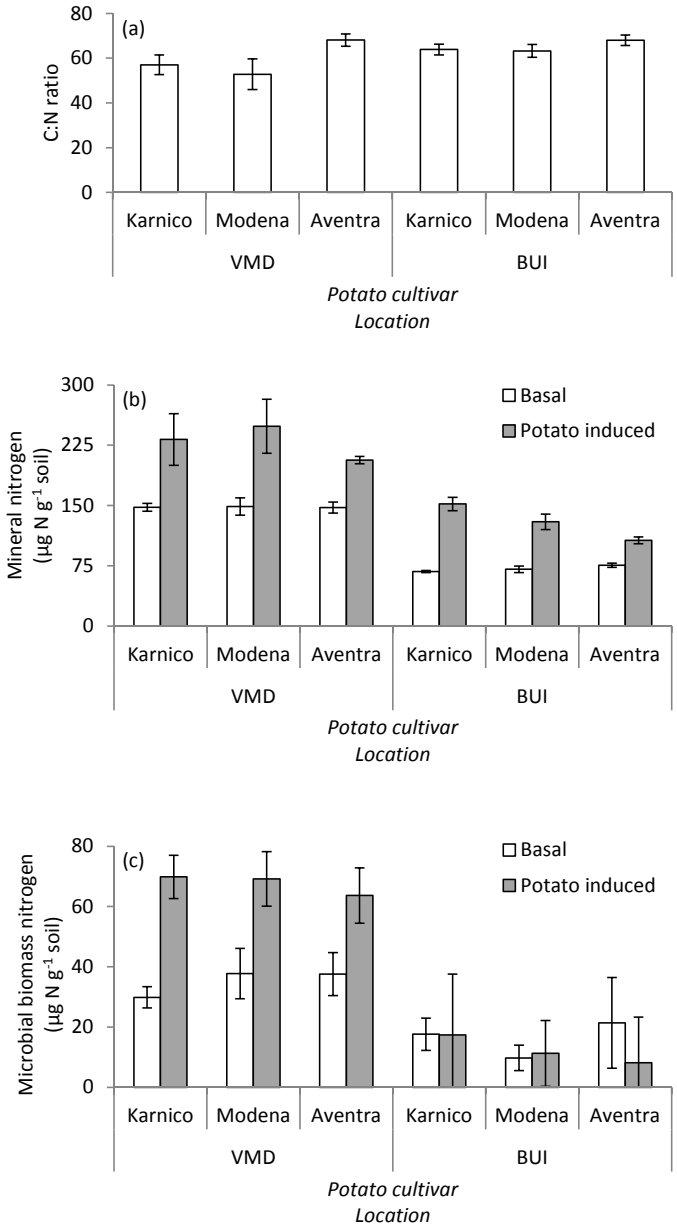


*Figure 5.2.*

Potato shoot C /N ratio (a) for potato cultivars Karnico, Modena and Aventra at location VMD and BUI. Error bars represent standard errors of the mean (*n* = 4). Mineral nitrogen (b) and microbial biomass nitrogen (c) in soil from the locations VMD and BUI after 121 incubation days with and without the addition of potato litter of from the cultivars Karnico, Modena and Aventra. Bars represent standard errors of the mean (*n* = 4).

Only location affected the decomposition processes at home, no effect of cultivar nor an interaction between cultivar and location were observed (Table 5.4). In general, decomposition was higher at location VMD compared to BUI. For C mineralization, the addition of potato material resulted in different mineralization rates between Aventra compared to Modena and Karnico for both locations (Fig. 5.3a & b). Mineral N increased after the addition of potato material at both locations (Fig. 5.2b). However, no increase in microbial N was observed after addition of potato material to the soil at location BUI (Fig. 5.2c).

*Table 5.4.*

The effects of cultivar, location and their interaction on basal and potato litter induced carbon mineralization, nitrogen mineralization and change in microbial biomass nitrogen based on ANOVA, significant results in bold ($n$ = 4).

| | | C mineralization | | N mineralization | | Microbial biomass N | |
|---|---|---|---|---|---|---|---|
| | | *F* | *p* | *F* | *p* | *F* | *p* |
| Basal | Cultivar | 0.13 | 0.88 | 0.12 | 0.89 | 3.08 | 0.08 |
| | Location | **631.40** | **<0.001** | **190.19** | **<0.001** | 3.20 | 0.09 |
| | C * L | 0.03 | 0.98 | 0.20 | 0.82 | 2.42 | 0.12 |
| Potato induced | Cultivar | 1.28 | 0.31 | 2.40 | 0.12 | 1.11 | 0.36 |
| | Location | **35.31** | **<0.001** | 2.10 | 0.17 | **12.93** | **0.003** |
| | C * L | 0.22 | 0.81 | 0.56 | 0.58 | 0.26 | 0.78 |

## *5.4 Discussion*

In this study, we determined the effects of the GM waxy starch potato cultivar Modena on soil biota and compared them with effects induced by the untransformed parental line and a conventional potato cultivar. No effects were observed of the GM cultivar Modena on microbial activities distinct from the effects induced by other potato varieties. Soil moisture was the main driver of the microbial catabolic diversity in bulk soil, and soil organic matter and sucrose content of potato tubers affected microbial catabolic activities in the rhizosphere. We observed a home field advantage of GM cultivar Modena in the decomposition experiment, but this advantage was found only in net N mineralization at location VMD. Similarly, the parental cultivar Karnico induced a home field advantage in net N mineralization at location BUI.

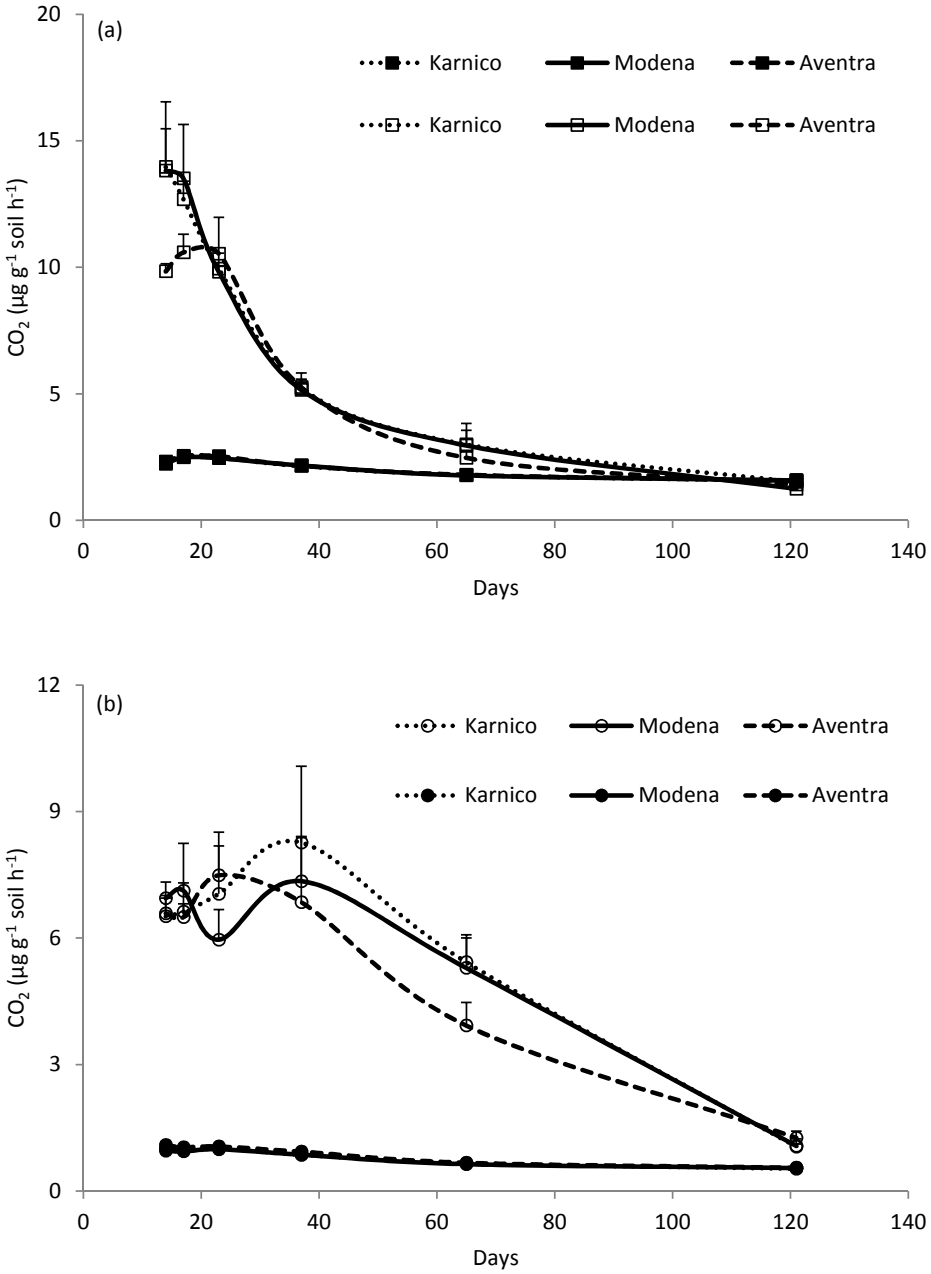


*Figure 5.3.*

Basal (closed symbols) and potato litter induced (open symbols) carbon mineralization from location VMD (a) and BUI (b) over the incubation period for potato cultivars Karnico, Modena and Aventra. Bars represent standard errors of the mean (*n* = 4; only positive direction of error bars shown).

A home field disadvantage was observed at both locations for cultivar Aventra. Decomposition of Aventra potato shoots resulted in lower soil mineral N during the incubation period at the home situation, as compared to Aventra shoots decomposing in Karnico and Aventra soils. Home field advantages or disadvantages can be explained by changing decomposer communities and altered litter quality (Aerts, 1997). We did not observed any differences in the catabolic response of the microbial community between cultivars at the start of the experiment. Hence, an explanation for the home field disadvantage of Aventra could be litter quality, seen that the C /N ratio is a good predictor of litter decay (Taylor *et al.*, 1989). However, litter quality was not affected by cultivar ($p$ = 0.07 and Fig. 5.2a). A possible explanation of the observed effect can be due to extra replications in the assessment of the home field advantage concept, which requires a reciprocal experimental set-up. This increases the number of replicas within the set-up, making a home field advantage assessment more robust to detect differences as compared to decomposition only at the home situation. However, we do not know whether the observed home field disadvantages were solely a matter of litter quality or of covarying interactions between litter and decomposer communities (Perez *et al.* 2013).

We observed a strong effect of location on microbial catabolic activities. We selected soil moisture and organic matter content as explanatory variables in the follow-up analysis (RDA). Organic matter content was selected because it is a resource for nutrients and energy, and it was the most distinctive between both locations with on average 25.5% organic matter at location VMD and 5.3% at BUI. Fierer *et al.* (2009) showed that broad scale structures of belowground microbial communities could be predicted by, among others, the soil C /N ratios. In addition, the soil physical environment influenced microbial eco-physiological performance and behaviour. Orchard and Cook (1983) observed that water potential showed a log-linear relationship with microbial activity, as long as activity was not limited by substrate availability. Our analysis showed that organic matter exerted the largest effects on microbial catabolic diversity in the rhizosphere, whereas in bulk soil moisture conditions explained most of the variation. This can be explained by the aspect that soil organic matter and soil moisture are tightly linked (e.g. Gupta and Larson, 1979 & Vereecken *et al.*, 1989), also we observed co-variations in the RDA between the two explanatory variables. The consequence of the tight linkages of moisture and organic matter in the RDA was that the variable that explained most of the variance (i.e. moisture in bulk soil microbial responses) resulted in low model improvement by inclusion of the other variable (organic matter). Nevertheless, this provides no explanation why the addition of similar substrate types resulted in opposite microbial responses. For example, the addition of glucose resulted in an inverse microbial response and association with soil organic matter in the rhizosphere as compared to sucrose induced effects (Fig. 5.1a). A possible explanation is that glucose is a monosaccharide, whereas sucrose (and trehalose) is a disaccharide and these different characteristics could affect microbial responses.

Rhizosphere microbial catabolic diversity was affected by tuber sucrose content. In a study by Back *et al.* (2010) sucrose stimulated growth of *Rhizoctonia solani*. Although a different potato cultivar was used in their study, it is possible that sucrose also entered the rhizosphere in our study as a potato leachate and affect microbial communities. However, in contrast to the findings of Back *et al.* (2010) microbial responses upon addition of various substrates did not resulted in straigtforward effects. So, assuming that increasing tuber sucrose content would also increase rhizosphere sucrose quantities it is likely that sucrose comprise selective effects on the rhizobiome.

## *5.5 Conclusions*

Based on the literature, we hypothesized that effects induced by GM cultivar Modena on soil biota and soil processes should be within the range of effects induced by conventional potato genotypic variation, in comparison to similar evidence for GM maize (Griffiths *et al.*, 2000 & Mulder *et al.*, 2006). We show that neither soil biota nor soil processes were affected to a detectable degree by the genetic modification. The results also demonstrate the relevance of including variation in soil characteristics while quantifying effects induced by cultivars. Including physical properties, like soil moisture, is recommendable in the belowground assessment of GM related effects. Our results further underline previous investigations on unexpected effects of Modena on soil biota and soil processes (Hannula *et al.*, 2012a,b & İnceoğlu *et al.*, 2012), all indicating that this particular GM waxy starch potato variety has either no measurable or consistent effects on soil systems (as in the case of nematodes in the same study area, see Vervoort, 2013). Based on the relevance of all these groups in terms of biodiversity, biomass distribution across trophic levels and food-web topologies, we tentatively conclude that the waxy starch GM potato cultivar Modena will not have significant effects related to its genetic modification. However, it must be underlined that the results of this study concern the effects of a single plant trait, i.e. the knock down of a specific carbohydrate metabolism pathway in a potato cultivar, and cannot be linked to other genetic modifications or translated to different GM crops.

# Chapter 6

## General discussion

Karst M. Brolsma

## 6.1 Introduction

Our understanding and appreciation of soil processes and biota are steadily increasing. Plant induced effects are investigated on belowground microbes, nematodes and related processes for this dissertation. The main findings from the experiments of the preceding chapters are summarized and discussed in this final chapter. The chapter is split in six sections based on four questions, a section on the MicroResp™ method, and conclusions. The aforementioned questions are: do our plant traits affect the microbial catabolic diversity in the rhizosphere? (6.2); are there linkages between microbial catabolic diversities in rhizosphere and bulk soil (6.3); do our plant traits affect microbial activities during litter turnover? (6.4); and, have the investigated plant traits consequences for nematodes in rhizosphere or in bulk soil? (6.6). In addition to the questions, section 6.5 is on the MicroResp™ methodology. For section 6.3, additional analysis were included for linkages between microbial activities in rhizosphere and bulk soil, and additional results were added from the field experiment of Chapter 3 for section 6.4. If possible, the findings are presented by starting at the plant species level, second at the level of plant genotypes and third at the level of plant traits. Suggestions are given for future research and the chapter ends with concluding remarks (6.7).

## 6.2 Do our plant traits affect microbial functioning in the rhizosphere?

### 6.2.1 Plant species effects on the microbial catabolic diversity

The grassland species in the long-term field experiment had distinct microbial catabolic diversities in the rhizosphere as measured with MicroResp™ (Chapter 2). Especially, the microbial catabolic diversities in the rhizosphere of *Rumex acetosa*, *Leucanthemum vulgare* and *Plantago lanceolata* were different as compared to the other plant species tested. The statistical assessment of the microbial response upon addition of substrates revealed that plant species separation was based on two organic acids, two amino acids and two carbohydrates. These findings suggest that the microbial communities in the rhizosphere were adapted to the substrates used in the MicroResp™ and could, possibly, reflect root exudation profiles of the plant species tested (Knox *et al*., 2014).

Lesuffleur *et al*. (2007) observed that root exudation of amino acids was plant species dependent. They found that two grasses (*Zea mays* and *Lolium perenne*) had lower amino acid outflow rates

as compared to two dicots (*Brassica napus* L. and *Lycopersicon esculentum* Mill.) and outflow of the amino acid glycine was much higher than for serine. Although plant growth conditions were not similar, a comparable response was found in Chapter 2. The four grasses resulted on average in lower rhizosphere microbial substrate induced respiration (SIR) as compared to the dicots, which could possibly be explained by lower exudation of amino acids. This is also supported by the finding that phenylalanine was the substrate in the MicroResp™ that had the strongest effect upon the separation of plant species. However, one of the grasses investigated (*Festuca rubra*) resulted also in high rhizosphere microbial SIR upon addition of phenylalanine. These findings suggest that there is not a general pattern in root exudation within plant families, under the assumption that rhizosphere measurements as done with the MicroResp™ reflect root exudation patterns.

### *6.2.2 Plant genotype effects on the microbial catabolic diversity*

In addition to plant species, also potato cultivars affected rhizosphere microbial catabolic activities (Chapter 2). Cultivar Aveka resulted in lower rhizosphere microbial SIR upon addition of carbohydrates than Aventra, Desiree and Karnico, but not than Modena. In contrast to these findings, no potato variety effects were detected in rhizosphere microbial catabolic diversity one year later in the same experimental field (Chapter 5). The dissimilar effects of potato varieties over these 2 years can potentially be explained by the lower number of cultivars assessed in 2011 as compared to the year before. However, a more likely explanation is that environmental factors exerted more pronounced effects on the potato varieties in 2011 as compared to 2010. Plant growth conditions between both years were different. Dry conditions were observed in 2010, whereas in 2011 precipitation and temperature were more constant over the year (Plischke, 2013). Such environmental conditions have their effects on plant growth and consequently on root exudation and microbial activities (Sanaullah *et al*., 2012). Likewise, Hannula *et al*. (2010) found that Aventra, Premiere and Désirée had lower enzyme activities in the rhizosphere than Karnico and Modena in 2008, but such effects were not consistent over 2009 or 2010.

So, no general patterns were observed related to induced effects of a single potato variety or genetically modified (GM) based effect. More often, a group of two or three cultivars differed from a cluster of the other potatoe cultivars in the biota that was assessed. For example, high starch containing cultivars (Aveka, Aventra, Karnico and Modena) clustered together, and were distinct from low starch containing cultivars (Désirée and Premiere) in terms of microbial community composition in the rhizosphere (İnceoğlu *et al.*, 2010). Although it is possible that the observed effects of potato cultivars (Hannula *et al.*, 2010 & İnceoğlu *et al.*, 2010) are due to altered root

exudation, e.g. low carbohydrates in rhizodeposits of Aveka and Modena in 2010 (Chapter 2), it is more likely that these potato varieties responded differentially to environmental conditions. Overall, these findings show that no single potato variety had distinct effects upon rhizosphere biota that was consistent over time as compared to the other potatoes.

### *6.2.3 Plant trait effects on the microbial catabolic diversity*

In Chapter 3, we included plant traits and soil characteristics in a statistical model to link these with microbial catabolic diversity in the rhizosphere. We found that root sinigrin (2-propenyl glucosinolate) level was associated with the microbial catabolic diversity. Although the amount of variation in microbial catabolic diversity data explained by sinigrin levels were not high, the observed effects were statistically sound. It is possible that more sinigrin in the roots resulted in higher respiration upon addition of various carbon (C) substrates in the MicroResp™. A possible explanation is that sinigrin was used as a substrate for C, nitrogen (N) and sulphur by rhizosphere microbes (Bressan *et al*., 2009 & Zeng *et al*., 2003). On the other hand, root herbivory by insects stimulates glucosinolate levels (Griffiths *et al*., 1996) and decreases levels of carbohydrates in *Brassica* roots (Pierre *et al*., 2012). Glucosinolates are involved in the defence system of plants and as such effects on the chemical composition of the roots could also be induced by plant pathogenic microorganisms (Diederichsen *et al*., 2014).

Future research can explore further how much sinigrin is released into the rhizosphere. Determining exudation rates of sinigrin in the rhizosphere and investigating factors that are involved in the hydrolysation of sinigrin to allyl isothiocyanate are key to untangle pathways of how microbial interactions in the rhizosphere affect, for example, the success of invasive plants species. In a study by Lankau (2012) it was observed that sinigrin levels of the invader *Alliaria petiolata* in forests of North America interacted belowground with spore germination of arbuscular mycorrhiza fungi (AMF). Without the invader, AMF stimulates growth of the native *Pilea pumila* and it was suggested that with increasing sinigrin level of the invader (*A. petiolata*) germination of AMF spores decreased. Spore germination of AMF *Glomus clarum* was inhibited by low levels of allyl isothiocyanate, the hydrolysed product of sinigrin (Cantor *et al.*, 2011). As a result, sinigrin levels had a role in the co-evolution of both plant species. Before the exotic species invaded, native *P. pumila* interacted only with one AMF species, whereas after the invasion *P. pumila* interacted belowground with several AMF species. Yet it is unclear through which pathway sinigrin interacts belowground; is it mainly through rhizodeposition or also through litter turnover?

## 6.3 *Are there linkages between microbial catabolic diversities in rhizosphere and bulk soil?*

Microbial catabolic activities were measured in rhizosphere and in bulk soil for various field experiments conducted with plant species (Chapter 2), mustard genotypes (Chapter 3) and potato cultivars (Chapter 2 and 5). Here in the current chapter, for each experiment case scores were calculated for the first axis of a principal component analysis (PCA) based on the microbial catabolic diversity in both soil zones, i.e. in the rhizosphere and in the bulk soil. Subsequently, Pearson's correlation coefficients were determined per experiment between the PCA case scores, to see whether linkages could be detected between microbial catabolic activities in the rhizosphere and bulk soil. Only in the potato cultivar experiment of 2011 a correlation was found between rhizosphere and bulk soil microbial catabolic activities was found (Table 6.1). In the corresponding PCA ordination plots of the potato experiment in 2011, similar microbial response patterns upon addition of substrates were found in the two soil zones (Fig. 6.1). Only addition of glucose resulted in distinct microbial responses in rhizosphere and bulk soil.

Potato cultivars stimulated microbial activities in the rhizosphere (higher SIR, Chapter 2), but did not have any consistent effect over 2010 and 2011 on the microbial catabolic diversities. So among the potatoes investigated, no selective effects of genotypes on microbial catabolic diversity were observed nor any distinct functioning of the microbes in rhizosphere as compared to the bulk soil. On the other hand, the plant species in the long-term experiment affected rhizosphere microbial catabolic diversities. The microbial catabolic diversity was furthermore distinct between the rhizosphere and the bulk soil (Table 6.1). These findings suggest that the functional profile of the microbes, as measured with the MicroResp™, differs between the rhizosphere and bulk soil due to plant species induced effects. Our findings indicate that over the relatively long period (>10 years), plant species selected in their rhizosphere microbes with distinct ecological characteristics from an initially equal bulk soil community.

The bulk soil microbial catabolic activities were also affected by plant species (Chapter 2). It was found that sucrose- and phenylalanine-induced respiration in the bulk soil was distinct for *L. vulgare* plots as compared to *Anthoxanthum odoratum*, *Festuca rubra* and *R. acetosa* plots. The observed effect of *L. vulgare* can be a result of dilution effects, i.e. bulk soil samples contained soil from the rhizosphere due to dense rooting systems. However, it is also possible that the observed effects were caused due to the possibility that *L. vulgare* litter turnover resulted in altered microbial catabolic activities in bulk soil. It is difficult to distinguish between these possible pathways based solely on the findings in Chapter 2. The comparable microbial response upon addition of sucrose

in both the rhizosphere and the bulk soil is in support of the dilution-effect. On the other hand, addition of phenylalanine had distinct effects in rhizosphere as compared to bulk soil, which suggests that the observed findings in the bulk soil cannot be explained by dilution only.

For both the mustard experiment and the potato experiment conducted in 2010, no correlations were found between the rhizosphere and bulk soil microbial catabolic profiles (Table 6.1). This does not necessarily mean that the microbial catabolic activities were affected differentially in these soil zones. Most likely, no correlation observed is due to smaller sample sizes and lower number of substrates that were used in the MicroResp™ for these experiments.

*Table 6.1.*

Pearson's correlation coefficients (*r*) as calculated for the case scores of the first PCA axis of rhizosphere and bulk soil microbial catabolic activities per experiment, it's significance (*p*), and the sample size (*n*).

| Experiment | Thesis chapter | *r* | *p* | *n* |
|---|---|---|---|---|
| Plant species | 2 | 0.07 | 0.63 | 44 |
| *Brassica juncea* | 2 | 0.18 | 0.45 | 20 |
| *Solanum tuberosum* 2010 | 2 | 0.39 | 0.16 | 15 |
| *Solanum tuberosum* 2011 | 5 | 0.82 | **<0.001** | 48 |

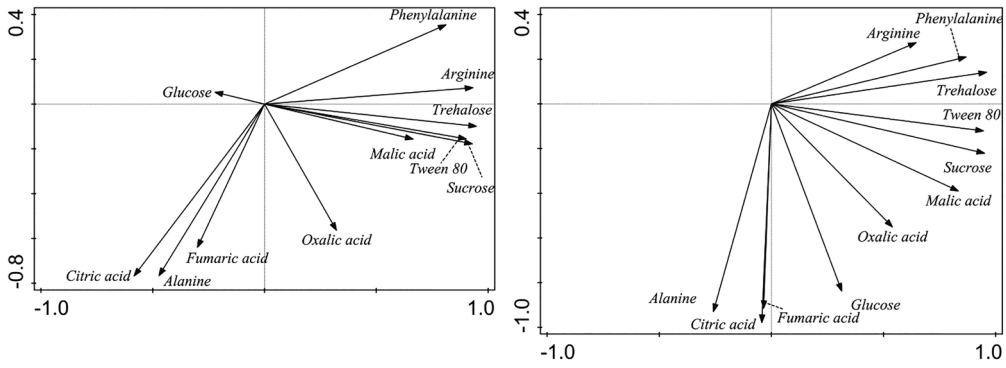


*Figure 6.1.*

Ordination diagram based on principal component analysis (PCA) of the microbial response upon addition of various substrates in the rhizosphere (left) and in the bulk soil (right) of the potato cultivar experiment in 2011, showing the first and the second axis accounting for 83% of the variance in the rhizopshere and 87% of the variance in bulk soil.

## 6.4 *Do our plant traits affect microbial activities during litter turnover?*

### 6.4.1 *Effects of plant genotype on microbial catabolic diversity during litter turnover*

In Chapter 3, we reported on rhizosphere effects of *Brassica juncea* genotypes, but in the same experiment also observations were made on after-crop effects on microbial catabolic activities in bulk soil. The *B. juncea* genotypes were macerated and incorporated into the soil 59 days after sowing (see for details Vervoort *et al.*, 2014) and microbial catabolic activities in the bulk soil were measured at 1, 22, and 57 days after incorporation (i.e. 60, 81, and 116 days after sowing) similar as in Chapter 3. It was found that mustard genotypes affected bulk soil microbial catabolic activities (Table 6.2). Decomposition of Terrafit resulted in higher substrate induced respiration upon addition of glucose, as compared to genotypes Terratop, Terraplus and ISCI 99 (Fig. 6.2). An explanation of the observed effects should be sought in the turnover of distinct litter qualities among the *Brassica* genotypes. However, measured levels of C, N, and indol glucosinolate of the mustard genotypes did not differ among the Brassicaceae at incorporation (Vervoort *et al.*, 2014). The only observed difference in litter quality was a higher calculated level of sinigrin in soil of one of the genotypes (ISCI 99) as compared to the other genotypes. So the litter characteristics measured were not good predictors of the microbial activities in the decomposer community.

*Table 6.2.*

The effects of *Brassica juncea* genotype, time and their interaction on the microbial catabolic diversity as measured with the MicroResp™ based on Wilks' Lambda (MANOVA). For each sample occasion (1, 22, 59 days after incorporation), the effects of *B. juncea* genotype are presented on the microbial catabolic diversity.

| | Time | Wilks'Lambda | *F* | *p* |
|---|---|---|---|---|
| Genotype (G) | - | 0.37 | 2.31 | **0.01** |
| Time (T) | - | 0.15 | 9.13 | **<0.001** |
| G * T | - | 0.32 | 1.28 | 0.18 |
| Genotype | T1 | 0.08 | 1.41 | 0.26 |
| | T22 | 0.02 | 2.74 | **0.03** |
| | T59 | 0.07 | 1.61 | 0.19 |

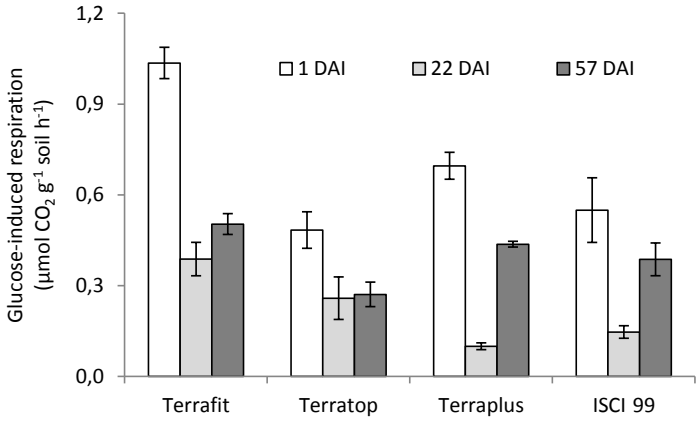


*Figure 6.2.*

Average glucose-induced respiration per *Brassica juncea* genotype for each sample occasion after plant incorporation into the soil, i.e. DAI is day after incorporation. Error bars are standard error of the mean, with *n* = 12.

## *6.4.2 Effects of plant traits on microbial activities during litter turnover*

Litter qualities were also taken into account in the decomposition experiment that was conducted in Chapter 5. This experiment revealed that shoot litter turnover of potato variety Aventra resulted in lower mineral N in its own soil, as compared to Karnico or Modena shoot litter mixed in soil previously grown by Aventra (a home field disadvantage for Aventra). Although we did not observe statistically significant differences among the cultivars in litter quality (C/N ratio), there was a tendency of higher C/N ratios in Aventra as compared to Karnico and Modena. Altered litter quality could have caused the observed effect in N mineralization, yet this was not confirmed by statistical analysis (Chapter 5). In addition, potato shoot metabolomic data were assessed in the same experimental plots by Plischke (2013). They found that Karnico and Modena showed strong overlap and were distinct from the other varieties in levels of organic acids and carbohydrates in the shoots. Although such compounds were the suggested drivers of the observed cultivar effects, no consistencies were found among the cultivars over time (Plischke, 2013).

## 6.5 *The MicroResp™ methodology*

The MicroResp™ provides a snapshot of microbial functioning (Campbell *et al*., 2003). Our results indicated that plants selected microbial communities with species specific functioning in their rhizosphere. The benefits of such plant specific microbial functioning in the rhizosphere needs further research. Another assumption made is that the MicroResp™ results reflect root exudation patterns. Also this assumption needs confirmation, especially for field situations in which roots experience other types of stress as compared to the root bathing methodology as was used e.g. by Lesuffleur *et al*. (2007).

Confirmation of the assumptions made could be done by using microsuction-cups in a rhizobox (for details see Duffner *et al*., 2012). As such, it is possible to measure glucosinolates and isothiocyanates in the rhizosphere and to link effects of root glucosinolate levels with rhizosphere concentrations and with microbial activities *in situ*. One could hypothesize that with increasing root sinigrin levels also sinigrin levels in the rhizosphere can be higher, which can stimulate microbial activity and might reduce pathogens (Chapter 3 & Kinkel *et al*., 2011). If higher levels of root glucosinolates have such benefits, these effects could have consequences for the disease suppressive status of a soil. The rhizobiome can be considered as a selection of the microbial community present in bulk soil (e.g. Sugiyama *et al*., 2013). On the other hand, the microbes in the rhizosphere will eventually become again part of the bulk soil (e.g. after root death or harvest) and these altered microbial communities will have legacy effects (Ter Horst *et al*., 2014).

Throughout this thesis, MicroResp™ results were linked with plant-induced effects. This work is part of the research about the effects of GM and non-GM crops on soil biota and ecosystem functioning. Effects of the GM potato cultivar Modena were explored in the rhizosphere (using the MicroResp™, Chapter 2 & 5) and during litter turnover (C and N cycling, Chapter 5). Modena was created by the knock-down of a specific carbohydrate metabolism pathway of the parental line (Karnico) (De Vetten *et al.*, 2003). This modification could affect rhizodeposit composition (e.g. carbohydrates) or litter quality. Various methods were used over several years for exploring the effects of potato varieties (including Modena) on belowground and aboveground biota (Hannula, 2012 & İnceoğlu, 2011 & Plischke, 2013 & Vervoort, 2013). Overall, factors that are not related to the modification (e.g. plant growth stage or soil type) had stronger effects on the measured biota. Moreover, the methodologies used for the assessment of Modena were sensitive to detect plant-induced effects (e.g. Chapter 3). Based on these findings, we can conclude that effects induced by Modena on biota and processes remained within the range of variation induced by conventional potato cultivars. So it seems that growing Modena tubers has little risks for soil ecosystems, an interesting result given

that GM-crops should pose no (or negligible) risks or impacts to man and environment. Without entering into the debate of defining "negligible", society needs to consider food production for 7 billion people and the question must be whether the biosphere can support human population. Actually, development, improvement, and introduction of novel crops, by conventional techniques and also by genetic modification, is already happening since historical times.

## 6.6 *Do our plant traits affect nematodes in rhizosphere and bulk soil?*

In Chapter 3, a correlation was found between *B. juncea* root glucosinolate levels and the nematode density. In addition, we found that hatching of *G. pallida* was strongly affected *in vitro* by allyl isothiocyanate (Chapter 4). Allyl isothiocyanate is the hydrolysed component of the main glucosinolate (sinigrin) in *B. juncea*. However, we did not find any decrease in hatching of *G. pallida* after growth of *B. juncea,* nor after incorporation of *B. juncea* plant material with increasing sinigrin levels.

Hatching of encysted *G. pallida* was taken as a measure for the effects of allyl isothiocyanate *in vitro*. We found that hatching of *G. pallida* was affected similarly in solution and in headspace by allyl isothiocyanate. However, the mode of action is not completely understood. Allyl isothiocyanate could mainly affect biochemical processes within the juveniles or it could also exerts effects upon the membranes surrounding the juveniles. Juveniles of *Globodera* spp. are affected by various types of isothiocyanates (e.g. Pinto *et al.*, 1998 & Serra *et al.*, 2002). It is generally accepted that isothiocyanate toxicity is due to an irreversible and nonspecific reaction with amino acids and proteins in organisms (Brown and Morra, 1997). For example, the expression of heat shock proteins in *Caenorhabditis elegans* was stimulated by allyl isothiocyanate as a stress response and could lead to excessive energy costs (Saini *et al.*, 2009). In addition, antimicrobial activities of allyl isothiocyanate were related to enzyme activities in microorganisms (Lin *et al.*, 2000 & Luciano and Holley, 2009). Next to that, Lin *et al.* (2000) observed that allyl isothiocyanate caused damage in the cell membrane leading to outflow of cellular metabolites.

Before any compound can interact with encysted *Globodera* spp. juveniles it has to cross the cyst wall and the eggshell. Valdes *et al*. (2011) observed that hatching of *G. rostochiensis* was stimulated more after the cysts were exposed to macerated *Brassica* leaf material and subsequent to tomato root exudates, as compared to cysts that were only exposed to tomato root exudates. The authors

suggested that, among others, the cause of the stimulated hatch was due to an increase in the permeability of the eggshell (Valdes *et al*., 2011), but this could not be confirmed due to technical issues (Valdes *et al.*, 2012b). It was hypothesized that the macerated *Brassica* material would release isothiocyanates, which is the basic concept of biofumigation. But if the concentration of isothiocyanates is not high enough as might have been the case in the study by Valdes *et al.* (2011), the compound instead stimulated hatching, but only in the presence of a host. So in practice the control of potato cyst nematodes through biofumigation with Brassicacea is only practically feasible if isothiocyanate levels in soil can be increased to lethal concentrations, preferably right after the harvest of the host plants of the nematode. However, in Chapter 4 we have suggested that unrealistic high biomass of the biofumigant is needed to control potato cyst nematodes. Increasing the amount of biofumigant and incorporation into the soil will also have consequences for beneficial soil organisms, since isothiocyanates have an nonspecific action on soil dwelling organisms (Brown and Morra, 1997 &b Lin *et al.*, 2000 & Luciano and Holley, 2009). As such, biofumigation with Brassicacea is not likely to be an acceptable solution in controlling potato cyst nematodes.

## *6.7 Conclusions*

The results presented in my dissertation confirm that plants comprise diverse and close associations with belowground biota and processes. Plant species in the long-term experiment shaped specific rhizosphere microbial communities that had distinct catabolic activities. In addition, also distinct microbial functioning was found between the rhizosphere and bulk soil. Furthermore, microbial catabolic responses in the rhizosphere were associated with belowground traits of potatoes and mustards. For potatoes, we did not observe any plant induced effects that could differentiate a GM cultivar from a selection of conventional cultivars. Finally, mustard litter turnover had its consequences for microbial catabolic activities, but it does not seem to be a practical method to control potato cyst nematodes.

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## *Summary*

A terrestrial plant can escape its environment through seed dispersal. Until that moment, a plant is attached to soil by its roots. Meanwhile a plant interacts with belowground biota and related processes through various plant specific traits. For this PhD-dissertation, belowground plant-induced effects were explored with the focus on rhizosphere and during plant litter turnover. Such knowledge is essential for risk assessment of genetically modified (GM) crops.

In Chapter 2, the effects of plant species and genotypes on microbial catabolic diversities were assessed in rhizosphere and in bulk soil. Four grasses and four herbs were compared in a long-term (>10 years) grassland experiment. In addition, four mustard (*Brassica juncea*) and five potato (*Solanum tuberosum*) genotypes were investigated during one growing season in agricultural field trials. Microbial activities were higher for most species in the rhizosphere as compared to the bulk soil. In the rhizosphere, microbial catabolic activities were plant species dependent and even potato genotype dependent. Furthermore, plant species affected also the microbial catabolic activity in the bulk soil differentially.

In Chapter 3, effects of *B. juncea* (Indian mustard) genotypes varying in root 2-propenyl glucosinolate (sinigrin) were investigated on biota in the rhizosphere. Root sinigrin appeared to be associated with microbial catabolic activities and with nematode density (i.e. total nematodes). In addition, total nematodes in the rhizosphere were associated with microbial responses upon addition of carbon rich substrates. It seems that sinigrin was used as a substrate that stimulated the rhizosphere biota.

In Chapter 4, the feasibility of controlling the potato cyst nematode *Globodera pallida* through biofumigation with *B. juncea* genotypes was assessed. Sinigrin is the main glucosinolate of *B. juncea* and hydrolysis of it through e.g. maceration of plant tissue (biofumigation) yields 2-propenyl (allyl) isothiocyanates. This is supposed to be toxic to *G. pallida*. It was found that hatching of *G. pallida* was reduced by exposure to allyl isothiocyanate. However, higher levels of the precursor of allyl isothiocyanate in *B. juncea* shoots did not resulted in a decrease in hatching of *G. pallida*. These findings suggest that it is unlikely to control potato cyst nematodes by *B. juncea* biofumigation.

In Chapter 5, the belowground impact of a GM potato cultivar on biota and related processes was assessed at two sample occasions and at two locations. A comparison was made between effects of the GM, its parental and a conventional cultivar on microbial catabolic responses in the rhizosphere and bulk soil in a field trial. In addition, effects of the three varieties were assessed on carbon and

nitrogen mineralization in a decomposition experiment. Mainly effects of location and time of sampling were detected and only minor effects of the potato genotype.

Overall, the results presented in this thesis confirm that plants have close and species dependent or even genotype dependent associations with the activities of belowground biota and related processes. The plant species in the long-term field experiment did not only affected microbial activities in the rhizosphere, but also in the bulk soil. In addition, plant traits were also associated with the rhizosphere microbial catabolic diversity and as are consistent with a role for secondary plant metabolites in selecting rhizobiome functioning. Although we found that allyl isothiocyanate had strong effects on the hatching of a potato cyst nematode *in vitro*, it is unlikely that *Globodera* spp. can be controlled through biofumigation using Brassicaceae. Finally, taken together our results with the findings of others on the assessment of a GM potato modified in its starch content, we can confirm that effects of this GM variety remains within genotypic variation on belowground biota and processes.

# *Samenvatting*

Een terrestrische plant kan zich alleen verplaatsen door zaadverspreiding. Totaan het moment van zaadverspreiding is een plant verbonden met de grond waarin deze staat. Door middel van specifieke eigenschappen hebben planten wisselwerkingen met de organismen en gerelateerde processen in de grond. Dit proefschrift gaat over plant-geïnduceerde effecten op organismen en processen in de rhizosfeer (een smalle zone rondom de wortel) en tijdens de afbraak van plantmateriaal. Inzicht in deze processen gerelateerd aan eigenschappen van planten is van belang, bijvoorbeeld in de risico analyse van genetisch gemodificeerde gewassen.

In Hoofdstuk 2 van dit proefschrift zijn de effecten van plantensoorten en plantgenotypen op de microbiële catabolische diversiteit bestudeerd in de rhizosfeer en in de bulk grond. Vier grassoorten en vier kruiden werden onderling vergeleken in een langlopend (>10 jaar) veldexperiment. Daarnaast zijn vier mosterdgenotypen (*Brassica juncea*) en vijf aardappelgenotypen (*Solanum tuberosum*) bestudeerd tijdens een groeiseizoen in een veldexperiment. De microbiële activitieit was hoger in de rhizosfeer ten opzichte van de bulk grond voor de meeste planten. Verder werden plantensoort en aardappelgenotype specifieke effecten gevonden in de microbiële catabolische diversiteit in de rhizosfeer. Daarnaast was de microbiële catabolische diversiteit in de bulk grond afhankelijk van de plantensoort.

In Hoofdstuk 3 zijn de effecten van verschillende *B. juncea* genotypen op organismen onderzocht in de rhizosfeer. Deze mosterdgenotypen hebben een verschillend gehalte aan 2-propenyl glucosinolaat (sinigrine) in de wortel. Uit de resultaten van dit veldexperiment bleek dat het gehalte aan wortelsinigrine geassocieerd was met de microbiële catabolische diversiteit en met de nematode aantallen in de rhizosfeer. Verder was het aantal nematoden gecorreleerd met de microbiële catabolische diversiteit. Waarschijnlijk wordt sinigrine in de *B. juncea* rhizosfeer gebruikt door de microben en de nematoden als bron voor koolstof, stikstof en zwavel.

In Hoofdstuk 4 is de mogelijkheid onderzocht om het aardappelcyste aaltje *Globodera pallida* te bestrijden door middel van biofumigatie met *B. juncea*. Sinigrine is een van de glucosinolaten van *B. juncea* en na hydrolyse, bijvoorbeeld door het verhakselen van de planten (biofumigatie), komt 2-propenyl (allyl) isothiocyanaat vrij. Allyl isothiocyanaat heeft toxische effecten op andere bodemorganismen. De resultaten van een toxiciteitsexperiment laten zien dat de wekking van *G. pallida* sterk afnam na blootstelling met allyl isothiocyanaat. Daarnaast was het mogelijk om het sinigrine gehalte hoger te krijgen in *B. juncea* door middel van bemesting en insectenvraat, maar een hoger gehalte aan sinigrine leidt niet direct tot een afname in het wekken van *G. pallida*.

Deze resultaten laten zien dat het waarschijnlijk niet mogelijk is om het aardappelcyste aaltje te beheersen door middel van biofumigatie met *B. juncea*.

In Hoofdstuk 5 zijn de (mogelijke) effecten onderzocht van een genetisch gemodificeerde aardappel op het bodemleven op twee locaties en op twee tijdstippen. Een vergelijking was gemaakt tussen de effecten van de genetisch gemodificeerde aardappel, de ouderplant en daarnaast een gangbaar aardappelras op de microbiële catabolische diversiteit in de rhizosfeer en in de bulkgrond in een veldexperiment. Daarnaast zijn de effecten van deze drie aardappelrassen gemeten op de koolstof en stikstof mineralisatie tijdens een decompositie experiment in het lab. Locatie en tijd hadden de grootste effecten op het bodemleven, daarnaast was er een cultivareffect gevonden welke niet consistent was.

De resultaten die gepresenteerd zijn in dit proefschrift bevestigen dat planten een nauwe en soorts en genotype afhankelijke associatie hebben met het bodemleven. De plantensoorten in het langlopende grasland experiment hadden niet alleen een effect in de rhizosfeer, maar ook in de bulkgrond. Daarnaast zijn plantspecifieke eigenschappen geassocieerd met de microbiële catabolische diversiteit in de rhizosfeer en deze eigenschappen zouden mogelijk een bijdrage kunnen leveren in de selectie van specifieke organismen in de rhizosfeer. We hebben aangetoond dat allyl isothiocyanaat toxische effecten heeft op de wekking van het aardappelcyste aaltje, het is waarschijnlijk niet mogelijk om deze te beheersen door middel van biofumigatie met Brassicaceae. Tot slotte, de analyse van de effecten van een genetisch gemodificeerde aardappel op het bodemleven in ons experiment, samen met de resultaten die gevonden zijn door andere onderzoekers, kunnen we concluderen dat de effecten die dit aardappelras induceert niet groter zijn dan de effecten van gangbare aardappelrassen.

# *概述*

陆地植物可以通过种子传播逃脱它自身的环境。直到那一刻，植物才通过根系与土壤黏贴在一起。同时，植物通过其各种各样的专一性特点与地下的微生物以及相关的过程进行交互作用。本论文针对在根际和根茬周转过程中植物所诱导的地下部影响进行了探索，其研究结果可为转基因作物的风险评估提供重要支撑。

第 2 章，主要评价植物物种和基因型对根际土和土体土中的微生物代谢活动的影响。研究对象包括长期（>10 年）的半自然试验田的四种禾本科草本和四种非禾本科草本植物；超过一个生长季节的四种芥菜（*Brassica juncea*）和五种马铃薯（*Solanum tuberosum*）基因类型的田间试验。结果表明：与土体土相比，大多数物种的微生物活动在根际范围内较高；在根际，微生物代谢多样性受到植物物种和马铃薯基因型的影响；此外，植物物种也能影响土体土中的微生物代谢多样性。

第 3 章，主要研究不同根系 2－丙烯基芥子油苷（黑芥子苷）水平的芥菜（印度芥菜）基因型对根际土壤微生物的影响。结果表明：根系黑芥子苷水平与微生物分解代谢活性以及线虫密度（即总线虫数量）相关；根际中总线虫数与富碳基质的添加引起的微生物反应密切相关。由此可知，黑芥子苷可作为刺激根际生物群的基质。

第 4 章，我们针对通过生物熏蒸芥菜（*B. juncea*）以控制马铃薯胞囊线虫（*Globodera pallida*）的可行性进行了评价。结果表明：黑芥子苷是芥菜中硫甙主要组成成分，且可通过水解（如生物熏蒸）产生 2-丙烯基和异硫氰酸盐，该成分对马铃薯胞囊线虫（*G. pallida*）有毒害作用；通过暴露在异硫氰酸烯丙酯中，马铃薯胞囊线虫（*G. pallida*）孵化减少了 50％；但是，更高水平的异硫氰酸烯丙酯的芥菜芽的前体并没有显著减少马铃薯胞囊线虫（*G. pallida*）的孵化。因此，通过生物熏蒸芥菜（*B. juncea*）可能无法针对马铃薯胞囊线虫（*G. pallida*）进行有效的控制。

第 5 章，基于两个取样时间和试验地点的结果，针对转基因的马铃薯品种影响地下土壤生物以及相关的过程进行了分析。在其中一个田间试验中，我们系统比较转基因品种、母本、传统品种对于根际土和土体土的微生物代谢活动的影响差异。此外，在实验室条件下，我们分析了这三个品种的根茬周转对于碳、氮矿化的影响。结果表明：取样时间和位置显著地影响着微生物的活性，但是马铃薯基因类型对其影响相对较小。

总体而言，本论文的研究结果证实植物与地下生物及其相关过程有着密切的和多样化的联系，这种联系会受到作物品种和基因型的影响。在长期试验田中，植物并没有仅仅选择其专一性的根际微生物的功能，同时根际与土体土的微生物的功能有较大差异。植物特点与根际微生物多

样性有密切关系，此特性支持了植物次生代谢产物选择根际生物功能。此外，虽然我们发现了异硫氰酸烯丙酯对孵化的体外马铃薯胞囊线虫具有强烈影响，但是利用生物熏蒸十字花科来控制该类型线虫孵化（*Globodera* spp.）的方式很可能行不通。通过结合该领域前人研究的结果，我们可以证实此转基因品种对地下微生物活动的影响仍然保持在基因型变异范围内。

“In a dark place we find ourselves...and a little more knowledge lights our way.”

Yoda

Star Wars - Episode III
Revenge of the Sith (2005)

# *Dankwoord*

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Freed de 15e fan ´e Rispmoanne, Wageningen.

# *Curriculum Vitae*

Karst Brolsma was born on the 11th of December 1981 in Leeuwarderadeel, The Netherlands. He finished his MSc in Plant Sciences in 2009, and worked as a researcher at the Grass Science Institute until he started his PhD in April 2010. In September 2014 Karst will start as a lecturer at the Mathematical and Statistical Methods Group.

**PE&RC Training and Education Statement**


With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

**Review of literature (4.5 ECTS)**

- Glucosinolates, Brassicaceae and soil ecology at the Julius Kuhn Institute Muenster, Germany

**Post-graduate courses (4.1 ECTS)**

- Soil ecology; PE&RC, E&E,SENSE (2010)
- Linear models; PE&RC (2011)
- Generalized linear models; PE&RC (2011)
- Mixed linear models; PE&R (2011)

**Laboratory training and working visits (1 ECTS)**

- Statistics; RIVM, Bilthoven (2011/2012)

**Invited review of (unpublished) journal manuscript (2 ECTS)**

- Applied Soil Ecology: earthworms, rhizobacteria, plant growth promoting bacteria, plant growth and development (2014)
- Plant and Soil: microbial diversity, microbial activity; Ralstonia solanacearum, tomato (2014)

**Deficiency, refresh, brush-up courses (0.3 ECTS)**

- Food web ecology and biodiversity (2010)

**Competence strengthening / skills courses (4.5 ECTS)**

- Scientific writing; Language Services (2013)
- Presentation skills; Language Services (2013)
- Career assessment; Meijer & Meijaard (2013)
- Career perspectives; Meijer & Meijaard (2013
- Ethics and philosophy; WIAS (2014)

**PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)**

- PE&RC Day (2011/2012)
- Introduction weekend (2010)

**Discussion groups / local seminars / other scientific meetings (4.5 ECTS)**

- Plant and Soil Interaction, Sustainable Intensification of Agricultural Systems (2012-2014)
- Local Seminars: Soil Quality, NIOO, CWE (2010-2014)
- Other meetings: WEES lectures, Wageningen Young Academy, NWO ERGO (2011-2014)

**International symposia, workshops and conferences (8.5 ECTS)**

- NERN; Lunteren (2011)
- Wageningen Conference on Applied Soil Science (2011)
- IRRS; Dundee (2012)
- Intecol; London (2013)

**Lecturing / supervision of practical's / tutorials (2.7 ECTS)**

- Soil-Plant interactions (2011-2014)

**Supervision of MSc students**

- J. Dogbatse: who is driving rhizosphere microbial activity: plants or soil? (2012)
- R. van der Salm: the effect of genotype and environment on glucosinolate levels in Indian mustard (*Brassica juncea*) and the subsequent effect on potato cyst nematodes (*Globodera pallida*) (2013)