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The effect of temporal variation in soil carbon inputs on interspecific plant competition

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Abstract

Aims

Release of carbon from plant roots initiates a chain of reactions involving the soil microbial community and microbial predators, eventually leading to nutrient enrichment, a process known as the 'microbial loop'. However, root exudation has also been shown to stimulate nutrient immobilization, thereby reducing plant growth. Both mechanisms depend on carbon exudation, but generate two opposite soil nutrient dynamics. We suggest here that this difference might arise from temporal variation in soil carbon inputs.

Methods

We examined how continuous and pulsed carbon inputs affect the performance of wheat (*Triticum aestivum*), a fast-growing annual, while competing with sage (*Salvia officinalis*), a slow-growing perennial. We manipulated the temporal mode of soil carbon inputs under different soil organic matter (SOM) and nitrogen availabilities. Carbon treatment included the following two carbon input modes: (i) Continuous: a daily release of minute amounts of glucose, and (ii) Pulsed: once every 3 days, a short release of high amounts of glucose. The two carbon input modes differed only in the temporal dynamic of glucose, but not in total amount of glucose added. We predicted that pulsed carbon inputs should result in nutrient enrichment, creating favorable conditions for the wheat plants.

Important Findings

Carbon addition caused a reduction in the sage total biomass, while increasing the total wheat biomass. In SOM-poor soil without nitrogen and in SOM-rich soil with nitrogen, wheat root allocation was higher under continuous than under pulsed carbon input. Such an allocation shift is a common response of plants to reduced nutrient availability. We thus suggest that the continuous carbon supply stimulated the proliferation of soil microorganisms, which in turn competed with the plants over available soil nutrients. The fact that bacterial abundance was at its peak under this carbon input mode support this assertion. Multivariate analyses indicated that besides the above described changes in plant biomasses and bacterial abundances, carbon supply led to an accumulation of organic matter, reduction in NO_3 levels and increased levels of NH_4 in the soil. The overall difference between the two carbon input modes resulted primarily from the lower total wheat biomass, and lower levels of NO_3 and soil PH characterizing pots submitted to carbon pulses, compared to those subjected to continuous carbon supply. Carbon supply, in general, and carbon input mode, in particular, can lead to belowground chain reactions cascading up to affect plant performance.

Keywords: competition, exudation, microbial loop, nutrient immobilization.

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INTRODUCTION

Current views in plant ecology consider plant competition as a highly complex process involving direct (e.g. Allelopathy; Callaway *et al.* 2004) and indirect (e.g. Mycorrhiza; Jones *et al.* 2004) biotic interactions, potentially influencing community dynamics (Wardle *et al.* 2004). An interesting example of such complexity is the interplay between plants and belowground microorganisms, which directly influence the soil nutrient pools, while indirectly influencing plant growth and competitive ability.

An important belowground process that can mediate the interaction between plants and soil microorganisms is plant

root exudation (Bardgett *et al.* 1998; Jones *et al.* 2004; Wang *et al.* 2012). Plants have been shown to exude a highly diverse array of substances into the rhizosphere, i.e., the narrow region of the soil that is directly influenced by root secretions and associated soil microorganisms (Bertin *et al.* 2003; Farrar *et al.* 2003; Nicholas 2007). Such root exudation has been shown to account for a significant fraction of plant fixed carbon, ranging from minute amounts (Farrar *et al.* 2003; Jones *et al.* 2004) up to significant carbon costs (Hamilton *et al.* 2001), which can be as high as 40% of the dry matter produced by plants (Lynch *et al.* 1990). The adaptive value of root exudation and its impact on nutrient cycling and plant–plant interactions has puzzled scientists for the last few decades (Bais *et al.* 2006; Bardgett *et al.* 2005; Bowman *et al.* 2004; Cheng *et al.* 2014; Clarholm 1985; Hamilton and Frank 2001; Klironomos 2002; Kuzyakov 2010; Perveen *et al.* 2014; Shahzad *et al.* 2015). This substantial contribution of carbon to the soil is especially interesting considering the nature of the interaction between plants and their associated microbial community (Keurentjes *et al.* 2011). While both need energy and nutrients in order to grow, each is dependent on the other to supply one of these requirements. On the one hand, while plants can produce their own chemical energy by photosynthesis, they are unable to mineralize soil organic matter (SOM), which is the main source of nutrients in the soil, and are dependent upon the microbial community's ability to do so. On the other hand, most of the soil microbial community is dependent upon plants as main suppliers of external energy sources in the form of carbon compounds found in leaf litter (Bowman *et al.* 2004), dead root material (Griffin *et al.* 1976) and in root exudates (Meier *et al.* 2009). Unlike root death and leaf defoliation, which are considered to be relatively inevitable processes, root exudation appears to be under a certain amount of plant control (Farrar *et al.* 2003).

Although both plants and soil microbes compete over available nutrients, by releasing carbon into the soil, plants supply energy-rich substrates needed for the growth of their microbial competitors (Cheng *et al.* 2014; Kuzyakov *et al.* 2001). Without energy limitation, the microbial community should be able to increase in size while immobilizing nutrients, enhancing the indirect negative effect on plant growth due to a lack of available nutrients. Bowman *et al.* (2004) hypothesized that plants that can survive in low nutrient conditions may exude carbon compounds to sustain the microbial community, causing nutrient immobilization and nutrient availability reduction, negatively affecting other plants with high nutrient demands. This strategy was suggested to be used by slow-growing plants, assisting them in preventing the fast-growing plants from establishing in their vicinity (Meier *et al.* 2009). For example, Eschen *et al.* (2006) illustrated that amending the soil with increasing levels of carbon resulted in decreased plant biomass accumulation that varied significantly between different plant species. Moreover, greater shoot reduction was found in fast-growing annual plants with high nutrient demands than in perennial species with low nutrient demands. Such a reduction in shoot biomass is indicative of nutrient stress, forcing the plants to invest more in their belowground rooting system at the expense of aboveground parts (Poorter *et al.* 2000).

The idea that plants exude carbon compounds to reduce nutrient availability in order to avoid competition with fast-growing neighboring plants, apparently contradicts the classical view regarding the adaptive value of plant exudation, namely, the 'microbial loop hypothesis' (Clarholm 1985). According to this hypothesis, plants release carbon compounds into the rooting zone to stimulate the growth of the microbial community. During this period of microbial growth, the microbial community immobilizes available nutrients in the soil. Consumption of the microbial community by soil microfaunal predators, such as protozoa and bacterivorous nematodes, remobilizes essential nutrients for plant growth. Grazing on the microbial community can have a positive effect on plant growth for several reasons, chief among them is that the microbial predators have relatively low assimilation efficiency, and therefore most of the nutrients immobilized by the microbial community are excreted back into the soil and are readily available for plants' uptake (Ferris *et al.* 1997). Alternatively, root exudation may initiate a series of bottom-up effects on the microbial community (Bonkowski 2004), beginning with an accelerated growth rate of fast-growing bacteria that quickly respond to increased carbon availability by increasing their activity and biomass. As a result, microfaunal predator recruitment increases, leading to a change in the soil community composition, selecting for microorganisms able to respond to enhanced grazing (e.g. filamentous bacteria cells; Hahn *et al.* 1999). Selective grazing of fast-growing bacteria enables the proliferation of other bacterial groups (Bonkowski *et al.* 2000, Griffiths *et al.* 1993, Verhagen *et al.* 1994), such as slow-growing SOM-decomposing groups (Henkinet *et al.* 1990), often resistant to predation (Fontaine *et al.* 2003). The two possibilities are not mutually exclusive and both may result in increased soil nutrient availability.

Both the nutrient immobilization and microbial loop hypotheses are based on the idea that plants utilize carbon exudation, but while in the former it leads to nutrient limitation in the latter it results in nutrient enrichment. We suggest here that this apparent contradiction between the 'microbial loop' and 'immobilization' hypotheses can be reconciled when considering the temporal variation in soil carbon inputs. We address two possible modes of carbon inputs: (i) Continuous and (ii) Pulsed. Each input pattern influences the soil microbial community in a different way and may hold a different outcome regarding nutrient availability, indirectly affecting the interaction between neighboring plants competing over available nutrients.

Continuous carbon input

When trace amounts of carbon enter the soil continuously the microbial community is expected to increase its biomass,

reaching certain equilibrium with the carbon supply rate. As long as the microbial community retains a constant biomass, it is assumed to efficiently recycle the carbon and nutrients released as a result of death and predation, thus restricting the growth of plants due to nutrient limitation.

Pulsed carbon input

In contrast to the above, pulsed carbon inputs can result in a temporal microbial release from the top-down control of predators, enabling fast microbial growth. During this growth period, nutrient limitation is expected since birth rates are higher than death rates. Microbial decline is expected to follow due to predator population growth (Anderson *et al.* 1978), predator spatial recruitment (Griffiths *et al.* 1993) and carbon exhaustion. During the phase of microbial decline, large amounts of nutrients are liberated as a result of both microbial death and predation (Bonkowski 2004). A significant fraction of these nutrients, which are either emitted directly into the soil or via predators (Ferris *et al.* 1997), are expected to be available for plants, since complete recycling into microbial biomass is not possible when microbial death rates exceed birth rates. Such community-level priming effects can occur over time scales of several days to weeks (Blagodatskaya and Kuzyakov 2008). For example, Wu *et al.* (1993) observed maximum CO_2 production attributed to priming effects peaking 10 days after glucose amendment. Hamer and Marschner (2005) showed that repeated substrate additions can lead to accelerating priming effects over a longer time period. Kuzyakov (2010) addressed pulsed carbon input as the addition of ready available soluble organics to the soil, leading to hotspots of microbial activity characterized by high turnover rates. A follow-up study estimated that such hotspots last only for a few days (Pausch and Kuzyakov 2011).

Indeed, in a previous experiment (Shemesh *et al.* 2015), examining the effects of temporal variation in soil carbon inputs on the performance of wheat plants growing alone, we demonstrated that pulsed carbon inputs, given at intervals of two to four days, can lead to increased reproductive allocation at the expense of decreased root allocation. Notably, such a positive effect on the performance of the fast-growing wheat plants was not evident when carbon pulses were given at 6- and 8-day intervals (Shemesh *et al.* 2015). The interplay between plants and the soil microbial community may also have an important role in shaping plant community dynamics (Bever 2003). Furthermore, Meier *et al.* (2009) suggested that carbon exudation could mediate the coexistence between fast-growing and slow-growing plants through changes in the microbial community. Here, we took the next logical step, examining the manner by which temporal variation in soil carbon inputs influences the strength of interspecific competition in plants.

Continuous carbon input might be rewarding for slow-growing plants in nutrient-rich environments, where they are often exposed to the risk of being out-competed by fast-growing plants (Bowman *et al.* 2004; Meier *et al.* 2009). Such an input mode is predicted to reduce nutrient availability in the vicinity of the slow-growing plant, preventing the establishment of fast-growing plants that have higher nutrient demands. On the contrary, Paterson and Sim (1999) found that defoliated plants tended to release carbon pulses from their roots and that the amount of carbon exuded was greater in nutrient-poor conditions. The fact that defoliated plants suffer severe nutrient shortage (due to biomass removal) agrees with our hypothesis, suggesting that pulsed carbon inputs may allow plants inhabiting nutrient-poor environments to increase nutrient availability through the 'microbial loop' pathway.

Instead of testing if plants can actually manipulate the rhizosphere via carbon exudation, we artificially manipulated the temporal variation in soil carbon inputs and tested for the effects on a wide range of variables, starting from soil chemical profile, through bacterial abundance and up to plant performance. To the best of our knowledge, this is the first attempt to explore the consequences of small-scale temporal variation in soil carbon inputs on the performance of plants experiencing competition.

METHODS

We carried out a fully factorial experiment designed to address different aspects concerning the soil carbon input modes. Specifically, we aimed at examining the combined effect of temporal variation in soil carbon inputs, SOM availability and nitrogen (N) supply on the performance of a fast-growing plant experiencing competition from a slow-growing plant. Each treatment combination was replicated 25 times (2 SOM levels × 3 carbon levels × 2 nitrogen levels × 25 replicates = 300 pots in total).

Model plants

Our model system included two plant species differing in their life history, as well as in their ability to tolerate nutrient shortage. Specifically, we used common wheat, *Triticum aestivum*, as our model organism representing a fast-growing, high nutrient demanding plant (Angus *et al.* 1985), while the slow-growing, low nutrient demanding plant was represented by the perennial common sage *Salvia officinalis*.

Soil preparation

Sand dune soil was collected from the hills outside Be'er Sheva, Israel (N 31°06′, E 34°49′; for a chemical description of the soil, see Singer 2007). Soil was sieved (<5 mm) and fine roots and other plant remains were removed. Different soil variables such as: soil pH, electrical conductivity, SOM content (%OM), and nitrogen as nitrate ($N\text{-}NO_3$) and as ammonium ($N\text{-}NH_4$) were measured prior to the experiment and were used when applicable as reference values of the soil (for more details see Soil chemical analysis).

Temporal variation in soil carbon inputs

Carbon treatment included the following two carbon input modes: (i) continuous, and (ii) pulsed. Carbon was added

as glucose (D-Glucose, Monohydrate, Duchefa Biochemie, NL), which is a simple carbon that was found in root exudates of almost all plants (Bertin *et al.* 2003; Farrar *et al.* 2003) and is utilized by many microbial species. *Continuous carbon input*: A daily release of minute amounts of glucose (0.015 g) through drip watering system. *Pulsed carbon input*: Once every 3 days, glucose (0.045 g) was added directly to each pot, using a pipette, resulting in a short release of high amounts of glucose compared to the continuous carbon supply treatment (the amount was three times greater). As for the justification of the selected time interval, see above discussion related to community-level priming effects. Note that the two carbon input modes differed only in the temporal dynamic of glucose, but not in total amount of glucose added. Moreover, water was provided through the watering system and was consistent among all three carbon treatments (i.e. no-carbon control, continuous carbon input, pulsed carbon input).

SOM availability

According to Fontaine and Barot's model (2005), decomposition rate is correlated with SOM content. In 'poor' soils, the SOM decomposition rate is expected to increase as a result of carbon addition, whereas in 'rich' soils this rate is assumed to remain low (but see Kuzyakov *et al.* 2000). Manipulation of decomposition rate was expected to affect the amount of available nutrients for plant growth; therefore affecting the wheat plants' performance. We used two types of soil treatments representing two levels of SOM availabilities. 'Rich' soil with high SOM content and 'poor' soil with low SOM content.

SOM-poor soil treatments: Potting material for the 'poor' treatment was comprised only of sandy soil with no additional organic matter.

SOM-rich soil treatment: Sandy soil amended with 50 ml sieved compost mixture (Garden Bio-compost, Deshanit, IL), which comprised about 1/8 of the pot volume; in both treatments the final soil volume was 400 ml.

Nitrogen treatments

Carbon and nitrogen are non-substitutional resources required for microbial growth, and their ratio in root exudates can strongly influence the microbial community (Hodge *et al.* 2000). We thus manipulated the solution's C:N ratio by adding nitrogen (KNO_3, potassium nitrate, Merck, DE). Specifically, when nitrogen was added, the C:N ratio in the added solution was adjusted to ~1, meaning continuous-supplied plants received a daily amount of 0.015 g nitrogen, and pulse-supplied plants received a triple amount of 0.045 g every three days. The control group received water only.

Plant harvest

At the end of the wheat-growing period (72 days), all plants were harvested and dismantled into: roots, vegetative shoot and reproductive parts. All plant parts were oven-dried and their dry biomass was determined.

Soil chemical analysis

At the end of the experiment, 50 ml tubes were filled with soil samples from three random blocks consisted of all treatment combinations. The samples were oven-dried in 60°C for 48 h, sealed in paper bags and then sent for soil chemical analysis at Gilat Hasade Services Laboratory (Moshav Gilat, Israel).

Soil chemical analyses were performed according to standard protocols for soil analyses (Sparks 1996): organic matter content by dichromate oxidation method, pH and electrical conductivity (EC) in saturated soil extract (SSE), phosphate by the 'Olsen method' (in sodium bicarbonate extract), nitrogen as nitrate in aqueous extract and nitrogen as ammonium in KCl solution extract (including adsorbed nitrogen).

Since we had the organic matter reference value of the soil at the beginning of the experiment, we were able to calculate the relative rate of change in SOM during the experiment. This relative rate of change was calculated using the following exponential function (Hunt 1982):

$$relative\,rate\,of\,change\ (day^{-1}) = \frac{ln\left(\frac{OM_{t=72}}{OM_{t=0}}\right)}{experimental\,days\,no. = 72\,days}.$$

Soil total bacteria

Soil samples were collected from three random blocks. The first block was sampled at the beginning of the experiment, when seedlings were relatively small (day 28), the second block was harvested in the middle of the experiment (day 52) and the third block on the last day of the experiment (day 72). Three, sterile, 50 ml tubes were filled with fresh soil from each pot. In the laboratory, the three samples of each pot were pooled together and homogenized. Triplicates, of 25 g each, of the homogenate were stored at −80°C for subsequent DNA extractions.

DNA extraction

DNA was extracted from the three replicates of soil homogenates using the PowerSoil™ DNA Isolation Kit (MoBio, West Carlsbad, CA, USA), according to the manufacturer's recommendations.

Quantitative (real-time) PCR

To measure the abundance of rRNA gene copies in the soil samples, we used real-time, quantitative PCR (qPCR). Primers Eub-341F (5′-CCTACGGGAGGCAGCAG-3′) and Eub-519R (5′-GWATTACCGCGGCKGCTG-3′) amplifying short fragments of 178 bps were used in order to quantify the 16S rRNA copy numbers of bacteria. A calibration curve was created using DNA extracted from *Escherichia coli*. For calibration, four different standard serial dilutions were amplified in parallel to the samples. The range of qPCR efficiency was between 0.96 and 1. Triplicates of 20 µl were used for each qPCR reaction containing: 10 µl of DyNAmo Flash SYBR Green mix (Finnzymes, Finland); 1 µl of 200 nM primers (Metabion); 5 µl of 10–20 ng μl^{-1} templates, and 4 µl of molecular grade

water (Sigma). Samples were run in a real-time PCR machine (Corbett, Australia) under the following conditions: 40 cycles of 95°C for 15 s, 63.3°C for 15 s, 72°C for 45 s. The relative abundance in each sample was calculated based on the calibration curves of the reference bacteria.

Statistical analyses

In order to minimize mean proportional variance differences, all mass and number data were log-transformed. An angular transformation ($\arcsin(\sqrt[2]{P_i})$) was used when analyzing proportions such as root and reproductive allocations. All presented data is non-transformed.

Plants

We used split-plot ANOVAs to analyze the combined effects of carbon input mode, SOM availability and nitrogen supply on plant total, root and shoot biomasses and allocations. Plant species was defined as the within-plot treatment and carbon input mode, SOM availability and nitrogen supply as whole-plot treatments. A significant treatment × species interaction, indicate that the two plant species respond differently to the treatment. That is, the advantage of using such a split-plot design is that it allows testing for quantitative differences between the two plant species in their responses to the experimental treatments. Similar species-specific responses were obtained when analyzing the data of each plant species separately; however, this approach allows only qualitative, rather than quantitative, comparison between the two plant species. Instead of using post-hoc tests and to minimize the number of pair-wise comparisons, we considered only the following two carbon treatment contrasts for each of the four possible SOM by nitrogen treatment combinations: (i) no carbon control versus carbon supply, and (ii) continuous carbon input versus pulsed carbon input.

Soil total bacteria

Soil total bacteria (number of gene copies per 1 g of soil) were analyzed using a nested ANOVA with carbon input mode, SOM availability and nitrogen supply as fixed explanatory variables. Since replicates of the same soil sample cannot be considered as independent of each other, they were nested within the three-way interaction term of the carbon, SOM availability and nitrogen supply treatments. Time during the growing season at which the soil samples were collected was also included in the model as a random factor.

Soil chemical analysis

We used three-way ANOVA to analyze the combined effects of carbon input mode, SOM availability and nitrogen addition on soil N-NO_3, N-NH_4, organic matter content and relative rate of change in SOM. Instead of using *post hoc* tests and to minimize the number of pair-wise comparisons, we considered only the following two carbon treatment contrasts for each of the four possible SOMs by nitrogen treatment combinations: (i) no carbon control versus carbon supply, and (ii) continuous carbon input versus pulsed carbon input.

Multivariate analysis

We Z-score transformed all of the data on the plants' total biomass, soil total bacteria and soil chemical characteristics (pH, EC, NO_3, NH_4, P, %OM) to create a set of per-pot profiles of these characters and used them in order to find dissimilarities resulting from N, SOM or carbon input mode.

Using the PRIMER v6 software (PRIMER-E Ltd, Plymouth, UK), we generated a dissimilarity matrix comprising the Euclidian distance of all the possible pairwise comparisons. Next, to find the 'natural groupings' of the different profiles—namely, when profiles within a group are more similar to each other compared to profiles in different groups—we used a non-metric multi-dimensional scaling (nMDS), followed by analysis of similarity (ANOSIM), testing for differences between the three carbon input modes. Using 'similarity percentages' routine (SIMPER), we assessed the contribution of each of the variables measured to the differences detected among soil dynamics.

We used multivariate analysis of variance (MANOVA) followed by canonical discriminate function analysis to test for differences in the Z-score transformed soil and plant features between the three carbon input modes. MANOVA enabled us to test for significant differences between the carbon input modes, while considering all plant and soil variables simultaneously. The second analysis enabled us to find the axis/axes, best separating between the three soil carbon input modes in the multi-dimensional space defined by all of these response variables.

RESULTS

Plants

Total biomasses

Carbon addition caused an overall reduction of 18% in the sage total biomass, while increasing the total wheat biomass by 7% ($F_{2,3}$ = 5.34, P < 0.01; see Supplementary Table 1 for the ANOVA results; Figs 1a and 2a), however, the interaction between carbon treatment and species was not significant (carbon treatment × species interaction; $F_{2,\ 203}$ = 0.66, P = 0.51). Nitrogen addition brought about a significant increase in the plant total biomass (64% and 42% increase for the wheat and sage plants, respectively; $F_{1,03}$=30.55, P < 0.001) and this pattern was consistent between the two plant species (N treatment × species interaction; $F_{1,203}$ = 0.089, P = 0.76). Interestingly, the combined effect of the carbon and nitrogen treatments was not consistent between the two plant species (carbon treatment × N treatment × species interaction; $F_{2,203}$ = 4.05, P = 0.018). Specifically, when nitrogen was added, carbon supply resulted in a 10% reduction in the total biomass of sage plants, while causing a 5% increase in the wheat total biomass. In contrast, when no nitrogen was added, there was a stronger negative effect of carbon supply on sage total biomass (16% reduction), while the positive effect on the wheat total biomass remained approximately the same (6% increase). The

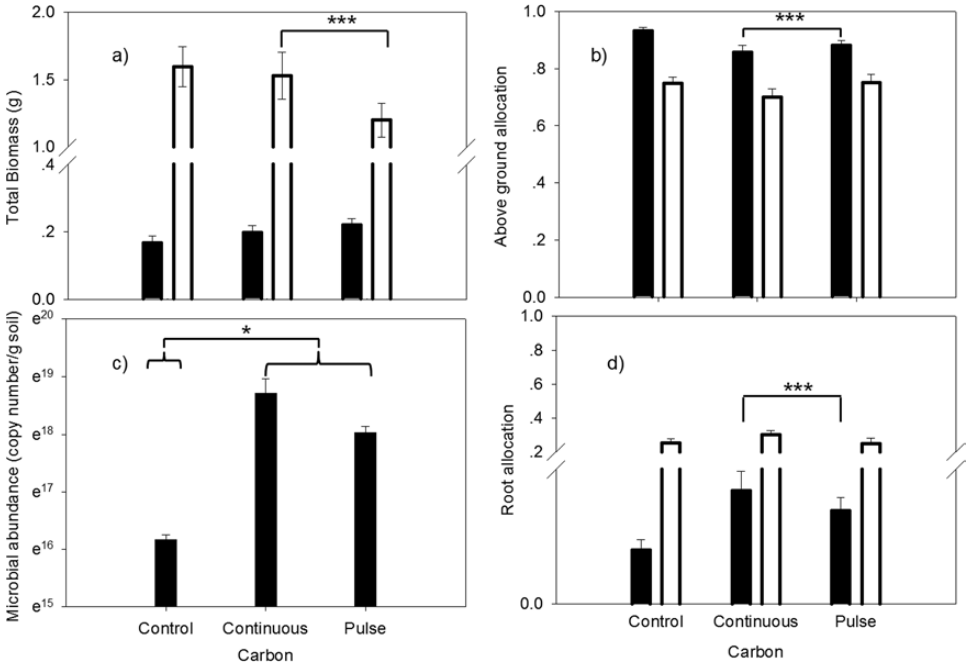

Figure 1: the effect of soil carbon input mode (no-carbon control, continuous, pulse) on plants (sage white bars, wheat black bars) grown in SOM poor soil without constant nitrogen supply: total biomasses (**a**), aboveground (**b**) and root (**d**) allocations; and on total soil bacterial abundance (c). Data are mean ± 1 SE. Carbon treatment contrasts; *0.01< P < 0.05, **0.001< P < 0.01, ***P < 0.001.

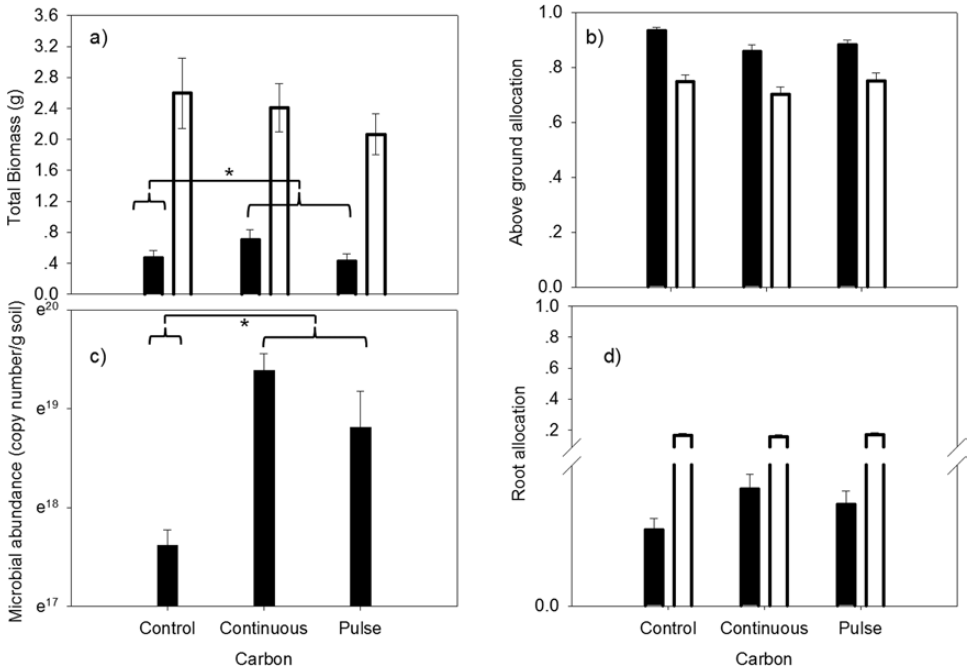

Figure 2: the effect of soil carbon input mode (no-carbon control, continuous, pulse) on plants (sage white bars, wheat black bars) grown in SOM rich soil with constant nitrogen supply: total biomasses (a), above-ground (b) and root (d) allocations; and on total soil bacterial abundance (c). Data are mean ± 1 SE. Carbon treatment contrasts; *0.01< P < 0.05, **0.001 < P < 0.01, ***P < 0.001.

carbon treatment contrasts indicated that wheat plants grown in SOM-rich soil supplied with nitrogen throughout the season had lower total biomass under carbon pulses than under continuous carbon input. In addition, when no nitrogen was added, carbon supply tended to reduce the total biomass of the sage plants grown either in SOM-rich or SOM-poor soil (See Table 1 for species-specific carbon treatment contrasts). Total biomass of plants grown in SOM-rich soil was

Table 1: Summary of two carbon treatment contrasts for the wheat and sage total, root and shoot biomasses under the four possible nitrogen by SOM treatment combinations (N = 1 with nitrogen, N = 0 no nitrogen, SOM = 1 SOM-rich soil, SOM = 0 SOM-poor soil): (i) no-carbon control versus carbon supply, (ii) continuous carbon supply versus carbon pulses

		Total biomass		Root biomass		Shoot biomass	
		No C control versus C supply	Continuous C supply versus C pulses	No C control versus C supply	Continuous C supply versus C pulses	No C control versus C supply	Continuous C supply versus C pulses
SOM = 0 N = 0	Wheat	$F_{1,176}$ = 1.67; P = 0.196	$F_{1,176}$ = 2.40; P = 0.123	$F_{1,176}$ = 0.02; P = 0.867	**$F_{1,176}$ = 12.5; P < 0.001; continuous > pulses**	*$F_{1,176}$ = 2.97; P = 0.086; control > C supply*	$F_{1,176}$ = 1.55; P = 0.214
	Sage	$F_{1,176}$ = 1.18; P = 0.278	*$F_{1,176}$ = 2.92; P = 0.089* continuous > pulses	$F_{1,176}$ = 1.20; P = 0.269	*$F_{1,176}$ = 3.35; P = 0.068; continuous > Pulses*	$F_{1,176}$ = 0.66; P = 0.415	$F_{1,176}$ = 1.63; P = 0.202
SOM = 0 N = 1	Wheat	$F_{1,176}$ = 1.01; P = 0.314	$F_{1,176}$ = 2.43; P = 0.120	**$F_{1,176}$ = 4.00; P = 0.046; C supply > control**	**$F_{1,176}$ = 5.40; P = 0.02; continuous > Pulses**	$F_{1,176}$ = 0.70; P = 0.403	**$F_{1,176}$ = 4.10; P = 0.042; continuous > pulses**
	Sage	$F_{1,176}$ = 2.27; P = 0.133	$F_{1,176}$ = 1.13; P = 0.288	$F_{1,176}$ = 1.76; P = 0.185	$F_{1,176}$ = 0.52; P = 0.471	$F_{1,176}$ = 2.10; P = 0.148	$F_{1,176}$ = 0.85; P = 0.357
SOM = 1 N = 0	Wheat	$F_{1,176}$ = 0.02; P = 0.879	$F_{1,176}$ = 1.35; P = 0.246	$F_{1,176}$ = 0.18; P = 0.667	**$F_{1,176}$ = 6.90; P < 0.01**	$F_{1,176}$ = 0.12; P = 0.719	*$F_{1,176}$ = 2.89; P = 0.090; pulses > continuous*
	Sage	$F_{1,176}$ = 0.71; P = 0.400	*$F_{1,176}$ = 3.28; P = 0.071;* Pulses > continuous	*$F_{1,176}$ = 2.84; P = 0.093; C supply> control*	$F_{1,176}$ = 1.32; P = 0.250	$F_{1,176}$ = 0.16, P = 0.683	**$F_{1,176}$ = 4.22; P = 0.041; continuous > pulses**
SOM = 1 N = 1	Wheat	**$F_{1,176}$ = 10.64; P = 0.001; C supply > Control**	$F_{1,176}$ = 1.32; P = 0.251	**$F_{1,176}$ = 6.1; P = 0.014**; C supply > Control	**$F_{1,176}$ = 6.80, P < 0.01 continuous > Pulses**	**$F_{1,176}$ = 11.5; P < 0.001; control > C supply**	$F_{1,176}$ = 0.66; P = 0.414
	Sage	$F_{1,176}$ = 0.64; P = 0.422	$F_{1,176}$ = 0.15; P = 0.694	$F_{1,176}$ = 0.20; P = 0.651	$F_{1,176}$ = 0.03, P = 0.862	$F_{1,176}$ = 0.65; P = 0.421	$F_{1,176}$ = 0.12; P = 0.724

Significant effects are marked in Bold and marginally non-significant effects appear in Italic

significantly higher than that of plants grown in SOM-poor soil ($F_{1,203}$ = 31.55, P < 0.001). However, this pattern was more prominent in the wheat than in the sage plants (73% and 19% increase for wheat and sage plants, respectively; SOM treatment × species interaction; $F_{1,203}$ = 15.0, P < 0.001).

Root biomasses

Nitrogen addition brought about reductions in root biomasses ($F_{1,203}$ = 18.22, P < 0.001) and this pattern was consistent between the two plant species (8% and 17% reduction for the wheat and sage plants, respectively; N treatment × species interaction; $F_{1,203}$ = 0.09, P = 0.762). Carbon addition caused an increase of 70% in the wheat root biomass, while causing a reduction of 10% in the sage root biomass (carbon treatment × species interaction; $F_{2,203}$ = 7.924, P < 0.001; see Table 1 for species-specific carbon treatment contrasts; see also Supplementary Table S1 for the ANOVA results).

Root allocations

Carbon addition caused an increase of 93% in the wheat root allocation, while causing a reduction of 8% in the sage root allocation (carbon treatment × species interaction; $F_{2,203}$ = 7.56, P < 0.001; see Supplementary Table S1 for the ANOVA results). Notably, when no nitrogen was added, the wheat root allocation of plants grown in SOM-poor soil tended to be higher under continuous carbon input than under carbon pulses. In addition, the sage root allocation of plants grown in SOM-rich soil without constant nitrogen supply was significantly higher under continuous carbon input than under carbon pulses (see Table 2 for species-specific carbon treatment contrasts; Figs 1d and 2d).

Nitrogen addition brought about significant reductions in root allocations ($F_{1,203}$=197.078, P < 0.001) and this pattern was more prominent in the wheat than in the sage plants (33% and 45% reductions for the wheat and sage plants, respectively; N treatment × species interaction; $F_{1,203}$ = 4.452, P = 0.036). Root allocation of plants grown in SOM-rich soil was significantly lower than that of plants grown in SOM-poor soil (42% and 11% reductions for the wheat and sage plants, respectively; $F_{1,203}$ = 52.164, P < 0.001), and this pattern was consistent between the two plant species (SOM treatment × species interaction; $F_{1,203}$ = 0.294, P = 0.58).

Shoot biomasses

Carbon supply caused an overall reduction of 12% in the sage shoot biomass, while increasing the shoot biomass of wheat plants by 1% ($F_{2,203}$ = 4.92, P = 0.008; see Supplementary Table S1 for the ANOVA results), but the interaction between carbon treatment and species was not significant (carbon treatment × species interaction; $F_{2,203}$ = 0.302, P = 0.74). Carbon supply caused significant reductions in the shoot biomasses in wheat plants grown in SOM-poor soil supplied with nitrogen and in sage plants grown in SOM-rich soil without constant nitrogen supply. Interestingly, in wheat plants grown in SOM-rich soil without nitrogen, carbon addition caused a significant increase in the shoot biomass. In addition, in wheat plants grown in SOM-poor soil without nitrogen, shoot biomass was higher when carbon was supplied in pulses rather than continuously (see Table 1 for species-specific carbon treatment contrasts). Shoot biomass of plants grown in SOM-rich soil was significantly higher than that of plants grown in SOM-poor soil ($F_{1,203}$ = 63.36, P < 0.001). However, this pattern was more prominent in the wheat than in the sage plants (92% and 29% increase for the wheat and sage plants, respectively; SOM treatment × species interaction; $F_{1,203}$ = 24, P < 0.001). Nitrogen addition brought about an increase in shoot biomass (78% and 74% for the wheat and sage plants, respectively; $F_{1,203}$=82.87, P < 0.001) and this pattern was consistent

Table 2: summary of two carbon treatment contrasts for the wheat and sage root and shoot allocations under the four possible nitrogen by SOM treatment combinations (N = 1 with nitrogen, N = 0 no nitrogen, SOM = 1 SOM-rich soil, SOM = 0 SOM-poor soil): (i) no-carbon control versus carbon supply, (ii) continuous carbon supply versus carbon pulses

		Root allocation		Above-ground allocation	
		No C control versus C supply	Continuous C supply versus C pulses	No C control versus C supply	Continuous C supply versus C pulses
SOM = 0 N = 0	Wheat	*$F_{1,176}$ = 3.01; P =0.084; C supply> Control*	**$F_{1,176}$ = 17.26; P < 0.001; Continuous>Pulses**	*$F_{1,176}$=3.01; P =0.084; Control>C supply*	**$F_{1,176}$ = 17.26; P < 0.001; Pulses>Continuous**
	Sage	$F_{1,176}$ = 0.69; P =0.404	$F_{1,176}$ = 1.88; P =0.171	$F_{1,176}$ = 0.69; P =0.404	$F_{1,176}$ = 1.88; P =0.171
SOM=0 N = 1	Wheat	$F_{1,176}$ = 2.51; P =0.114	**$F_{1,176}$ = 8.18; P < 0.01; continuous > pulses**	$F_{1,176}$ = 2.51; P = 0.114	**$F_{1,176}$ = 8.1; P < 0.01; pulses > continuous**
	Sage	$F_{1,176}$ = 0.06; P = 0.797	$F_{1,176}$ = 0.03; P = 0.848	$F_{1,176}$ = 0.06; P = 0.797	$F_{1,176}$ = 0.03; P = 0.848
SOM = 1 N = 0	Wheat	$F_{1,176}$ = 1.09; P = 0.295	**$F_{1,176}$ = 15.4; P < 0.001; continuous > pulses**	$F_{1,176}$ = 1.09; P = 0.295	**$F_{1,176}$ = 15.39; P < 0.001; Pulses > continuous**
	Sage	**$F_{1,176}$ = 3.93; P = 0.048; C supply> Control**	$F_{1,176}$ = 0.58; P = 0.444	**$F_{1,176}$ = 3.93; P = 0.048; C supply> Control**	$F_{1,176}$ = 0.58; P = 0.444
SOM = 1 N = 1	Wheat	$F_{1,176}$ = 0.22; P = 0.635	*$F_{1,176}$ = 2.91; P = 0.089; continuous > Pulses*	$F_{1,176}$ = 0.22; P = 0.635	*$F_{1,176}$ = 2.91; P = 0.089; pulses > continuous*
	Sage	$F_{1,176}$ = 0.03; P = 0.861	$F_{1,176}$ = 0.001; P = 0.967	$F_{1,176}$ = 0.03; P = 0.861	$F_{1,176}$ = 0.001; P = 0.967

Significant effects are marked in Bold and marginally non-significant effects appear in Italic.

between the two plant species (N treatment × species interaction; $F_{1,203} = 0.235$, $P = 0.62$).

Aboveground allocations

Carbon supply caused significant overall reductions in aboveground allocations, but this pattern was stronger in the wheat plants (5% and 1% reductions for the wheat and sage plants, respectively; carbon treatment × species interaction; $F_{2,203} = 7.57$, $P < 0.001$;see Supplementary Table S1 for the ANOVA results). Notably, in wheat plants grown in SOM-poor soil without constant supply of nitrogen, aboveground allocation was higher under carbon pulses than under continuous carbon input. A similar pattern was detected among sage plants grown in SOM-rich soil and without nitrogen (see Table 2 for species-specific carbon treatment contrasts; Figs 1b and 2b). Nitrogen addition brought about increased aboveground allocations ($F_{1,203} = 197.08$, $P < 0.0001$) and this pattern was more prominent in the sage than in the wheat plants (7% and 16% increase for the wheat and sage plants, respectively; N treatment × species interaction; $F_{1,203} = 4.45$, $P = 0.03$). Aboveground allocation of plants grown in SOM-rich soil was significantly higher than that of plants grown in SOM-poor soil (5% and 6% increase for the wheat and sage plants, respectively; $F_{1,203} = 52.16$, $P < 0.0001$), and this pattern was consistent between the two plant species (SOM treatment × species interaction; $F_{1,203} = 0.29$, $P = 0.58$).

Soil total bacteria

Carbon addition caused a one order of magnitude increase in the bacterial 16S rRNA gene copies number ($F_{2,44} = 26.36$, $P = 0.018$; see Supplementary Table S2 for the ANOVA results; Figs 1c and 2c). When contrasting only the continuous and pulsed carbon inputs, no significant effect of carbon on bacterial abundance was detected ($F_{1,44} = 1.19$, $P = 0.28$).

Soil chemical analysis

In pots constantly supplied with nitrogen, carbon addition, in general, and carbon pulses, in particular, brought about decreased soil nitrate. Such changes in nitrate availability were not evident when no nitrogen was supplied (carbon treatment × N treatment interaction; $F_{1,22} = 16.15$, $P < 0.0001$; see Supplementary Table S3 for the ANOVA results; also see Supplementary Table S4 for specific carbon treatment contrasts). Carbon pulses increased ammonium levels in pots amended with nitrogen (carbon treatment × N treatment interaction; $F_{2,22} = 3.24$, $P = 0.05$; see Supplementary Table S3 for the ANOVA results; also see Supplementary Table S4 for specific carbon treatment contrasts). Higher levels of ammonium were evident when the soil was amended with either SOM ($F_{1,22} = 14.20$, $P = 0.001$) or nitrogen ($F_{1,22} = 85.52$, $P < 0.001$). In addition, in pots supplied with nitrogen, continuous carbon input caused increased accumulation of the SOM (relative rate of change in SOM > 0). However, when no nitrogen was supplied, such a positive effect was evident only in pots subjected to carbon pulses (carbon treatment × N treatment interaction; $F_{2,22} = 5.23$, $P = 0.013$; see Supplementary Table S3 for the ANOVA results; also see Supplementary Table S4 for specific carbon treatment contrasts).

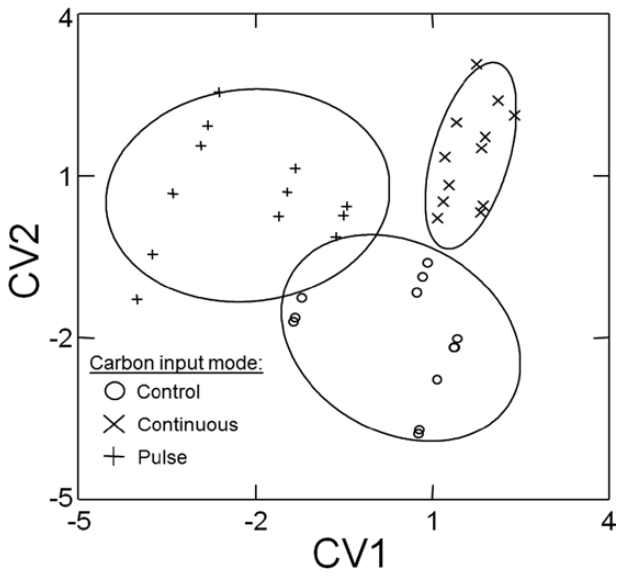

Figure 3: canonical discriminate analysis, pointing at the two orthogonal axes (CV1, CV2) best separating between the three different carbon input modes (Control: red, Continuous: blue, Pulse: green) in the multi-dimensional space defined by the following response variables: wheat and sage total biomasses, soil total bacteria, soil pH, EC, ammonium, nitrate and organic matter content. CV1 and CV2 explained 73.1% and 69.7% of the variation in the grouping of the carbon input modes, respectively.

$CV1 = 0.185 TotBac + 1.129 pH - 12.178 EC + 5.919 NO_3 - 3.369 NH_4 - 1.471 P + 7.545 OM - 1.723 WheatBM + 2.074 Sage\_BM$

$CV2 = 0.586 TotBac - 1.149 pH + 7.288 EC - 1.523 NO_3 + 1.45 NH_4 + 8.419 P - 11.062 OM - 0.907 WheatBM - 0.942 Sage\_BM$

Multivariate analysis

As expected, non-metric multi-dimensional scaling (nMDS) (based on the following variables: wheat and sage total biomasses, soil total bacteria, soil pH, EC, ammonium, nitrate and organic matter content) illustrated a clear separation between the nitrogen and SOM treatments. Using ANOSIM, we also detected significant differences resulting from the carbon input modes (Global $R = 0.204$, $P = 0.012$). Continuous carbon input had a significantly different profile than pulsed carbon input ($R = 0.233$, $P = 0.025$), while pulsed carbon input differed from the no-carbon control profile ($R = 0.175$, $P = 0.054$). SIMPER results showed that these differences between the no-carbon control and the carbon supply treatments (continuous and pulsed carbon inputs combined) resulted not only from biotic changes in the total sage and wheat biomasses, the bacterial gene copies number and the percent of organic matter in the soil, but also from abiotic changes in the chemical composition of the soil like changes in ammonium and nitrate levels. Specifically, carbon supply led to decreased total sage biomass, increased total wheat biomass, increased bacterial gene

copies number, an accumulation of organic matter, decreased NO_3 levels and increased levels of NH_4 in the soil. The differences between the continuous and pulsed carbon input modes resulted primarily from changes in soil pH, NO_3 and the wheat total biomass. Specifically, in pots submitted to pulsed carbon inputs, we observed lower total biomass of the wheat plants and lower levels of NO_3 and soil PH compared to the continuous carbon input (see Supplementary Table S5). These results were supported by MANOVA, indicating significant differences between the three soil carbon input modes in the multi-dimensional space defined by all of the above-described response variables (Wilks's Lambda = 0.082, Approx. $F_{18, 50} = 6.9$, $P < 0.001$), followed by a canonical discriminate analysis, clearly pointing at two orthogonal axes best separating between the different carbon input modes (see Supplementary Table S5; Fig. 3). The classification results reveal that 67% of the profiles were correctly classified into no-carbon-control, 100% into continuous carbon supply, and 83% into pulsed carbon supply groups. These three classifications were larger than those expected by chance (33%, three carbon treatment levels) by more than 25%, suggesting a reliable classification.

DISCUSSION

We examined the combined effect of temporal variation in soil carbon inputs, SOM availability and nitrogen addition on the performance of a wheat plant experiencing competition from a sage plant. It has been previously recognized that the addition of carbon to the soil may have far reaching effects on plant growth (Eschen *et al.* 2006). Here we show for the first time that besides carbon amounts, also the carbon input mode has a distinct effect on plant growth. Specifically, carbon supply led to an overall reduction in total sage biomass, increased total wheat biomass, increased bacterial abundance, an accumulation of organic matter, decreased NO_3 levels, and increased levels of NH_4 in the soil. The overall difference between the two carbon input modes resulted primarily from lower total wheat biomass, and lower levels of NO_3 and soil PH, detected in pots submitted to carbon pulses compared to those subjected to continuous carbon supply. These findings support our assertion that carbon supply, in general, and carbon input mode, in particular, can lead to belowground chain reactions cascading up to affect plant performance.

In SOM-poor soil and without constant nitrogen supply, carbon addition tended to reduce the total biomass of the sage plants, while increasing the wheat total biomasses. Contrary to our predictions, carbon pulses have not led to significantly improved performance (decreased root allocation or increased aboveground allocation) of the wheat plants grown in SOM-poor soil. Moreover, there was increased SOM accumulation in these pots, probably due to increased soil bacteria abundance. One possible explanation for the lack of clear improvement in the performance of the wheat plants is that, owing to increased bacterial abundance, they experienced a stronger competition from the soil bacteria.

In SOM-rich soil amended with nitrogen, wheat plants showed greater total biomasses under a continuous carbon input. Even though the total biomass of the wheat plants was higher, more biomass was allocated to the root system than to the aboveground plant parts. This allocation shift from shoot to the root system is a common response to reduced nutrient availability (e.g. Jongejans *et al.* 2006), suggesting that carbon supply caused nutrient limitation. We thus suggest that carbon supply stimulated the proliferation of soil microorganisms, which in turn competed with the plants over available soil nutrients, altering the plants' growth.

Hodge *et al.* (2000) argued that microbes–plant competition could be intense even in N-rich soil, since the speed at which new roots can be produced will never match the turnover rates of the microbial cells. In SOM-rich soil amended with nitrogen, continuous carbon input facilitated the accumulation of SOM. This is probably due to increased bacterial abundance in the soil. Indeed, under the same soil conditions combined with a continuous carbon input, soil bacterial abundance was at its peak. These results support Bowman *et al.* (2004) hypothesis suggesting that nutrient immobilization is inflicted by plant carbon compounds.

According to Bowman *et al.* (2004), a slow-growing plant should be able to compete with a fast-growing competitor by stimulating the microbial community, which can efficiently immobilize soil nutrients, and thus indirectly restrict the growth of the fast-growing neighboring plant. Thus, the interaction between slow-growing and fast-growing plants is highly dependent upon the slow-growing plant root exudation pattern. Klumpp *et al.* (2009) suggested that the plant community of slow-growing plants reduces the abundance of Gram-positive decomposing bacteria through their living root system, causing a reduction in decomposition rates of organic matter. Slow decomposition rates lead to low nutrient availability. This is preserved as a strategy assisting slow-growing plants to hinder the ingress of fast-growing competitors. In our study, we have not explored the root exudation patterns of the slow-growing plants; rather we have artificially manipulated the environment of both plant species. Our findings strongly suggest that in SOM-rich soils amended with nitrogen, carbon can cause nutrient immobilization, which is strong enough to restrict the growth of both plant species.

According to the 'microbial loop' hypothesis (Clarholm 1985), plants release carbon into the rooting zone to stimulate the growth of the microbial community. During this period of microbial growth, large amounts of nitrogen from organic matter are mineralized (a process plants cannot perform on their own), and immobilized by bacteria. This nitrogen is later liberated from the microbes and made locally available for uptake by plants due to the strong top-down regulation inflicted upon the microbial community by microbial predators (Bonkowski 2004; Moore *et al.* 2003). Our main working hypothesis was that carbon pulses should cause fluctuations in microbial abundance, resulting in nutrient enrichment that should positively affect the performance of the fast-growing wheat plant. Indeed, in SOM-poor soil without

constant nitrogen supply, common wheat tended to perform better under carbon pulses than under continuous carbon supply, i.e. higher total biomass, and higher shoot biomass and allocation.

In SOM-rich soil constantly supplied with nitrogen, carbon pulses led to reduced levels of soil nitrate, while increasing ammonium levels, compared to the no-carbon control and to the continuous carbon input mode. Since microbial predators have low assimilation efficiency, increased ammonium levels is a common outcome of microbial predation (Clarholm 1985). These results support our hypothesis that carbon pulses could lead to a chain of belowground reactions resulting in increased nutrient availability. Alternatively, high ammonium levels may also result from dissimilatory nitrate reduction, occurring under anaerobic conditions associated with high bacterial abundance. Although we observed increased nutrient availability under these conditions, we could not detect improved performance of the fast-growing wheat plants. It is possible that owing to the changes in the soil nutrient availability also the microbial community has changed, and if the emerging microbial groups have fast turnover times, they better competed with the wheat plants over available nutrients.

Although our study shows that temporal variation in soil carbon inputs can play an important role in a variety of ecological processes above and below the ground, there is still a lot to be done in order to strengthen the foundations of this complex hypothesis. One approach is to pursue the same methodology of artificially manipulating the soil carbon inputs and studying the effects on soil microbial community structure, by either looking for specific functional groups or by looking for overall changes in the microbial community profile under different temporal dynamics and SOM availabilities. Another appealing approach is to examine the effects of naturally occurring temporal root exudation patterns on soil nutrient cycling and plant performance. This can be achieved by conducting continuous 'Carbon pulse-chase' experiments. Such experiments should monitor variables such as pulse pattern (amplitude, frequency and duration) and composition. Combining both approaches will improve our grasp of the mechanisms linking plant competition and root exudation patterns to microbial dynamics and nutrient cycling, strengthening the link between two ecologically pivotal processes that have traditionally been addressed separately.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology* online.

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