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SCIENCE FOR A SUSTAINABLE DEVELOPMENT

MYCORRHIZAE IMPACT ON BIODIVERSITY AND C-BALANCE OF GRASSLAND ECOSYSTEMS UNDER CHANGING CLIMATE

"MYCARBIO"

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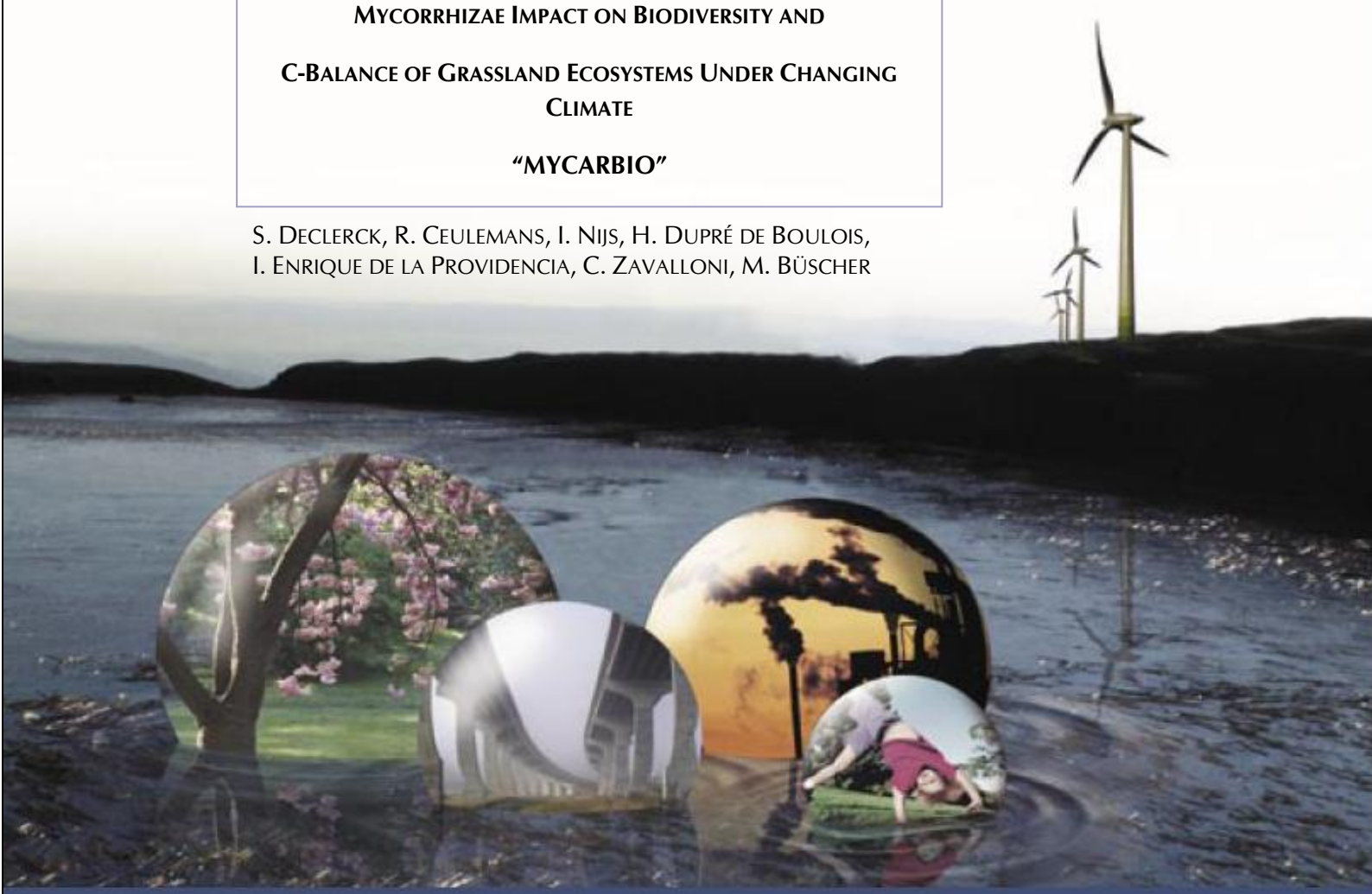

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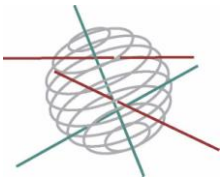

Biodiversity

FINAL REPORT

MYCORRHIZAE IMPACT ON BIODIVERSITY AND C-BALANCE OF GRASSLAND ECOSYSTEMS UNDER CHANGING CLIMATE

"MYCARBIO"

SD/BD/05

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Janvier 2009

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Universiteit Antwerpen

D/2012/1191/23
Published in 2012 by the Belgian Science Policy
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Stéphane Declerck, Reinhart Ceulemans, Ivan Nijs, Liesbet Voets, Hervé Dupré de Boulois, Ivan Enrique de la Providencia, Costanza Zavalloni, Manu Büscher. ***Mycorrhizae impact on biodiversity and C-balance of grassland ecosystems under changing climate "MYCARBIO"*** Final Report. Brussels : Belgian Science Policy 2012 – 67 p. (Research Programme Science for a Sustainable Development

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ACRONYMS, ABBREVIATIONS AND UNITS

AMF	arbuscular mycorrhizal fungi
Anafore	ANAlysis of FORest Ecosystems
C	carbon
IPCC	Intergovermental Panel on Climate Change
N	Nitrogen
P	phosphorous
SBSTTA	Subsidiary Body on Scientific, Technical and Technological Advice

SUMMARY

Climate change has and will continue to have profound effects on the structure and function of terrestrial ecosystems. It is thus imperative to improve our understanding of the complex response of ecosystems to climate change in order to enhance the scientific basis for national and international policies regulating carbon sequestration and greenhouse gas emissions. Improved management of grassland has been identified as a potential tool to combat climate change by enhancing carbon sequestration in soils but also in vegetation, while conserving biodiversity. Several recent studies have demonstrated the fundamental role played by above- and belowground communities in controlling ecosystem processes and properties. Thus, understanding the linkages between these communities under climate change (including increasing CO_2 concentration) will bring new insights on how communities and biological processes will evolve under future climate scenario.

MYCARBIO aimed to investigate the impact of arbuscular mycorrhizal fungi (AMF) on biodiversity and C cycle in Belgian grassland ecosystems under changing climate conditions. To achieve this objective, five specific tasks were identified:

(1) Evaluation of AMF biodiversity in selected Belgian grasslands,
(2) Determination of the role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands and their feedbacks on AMF,
(3) Understanding of the impact of elevated CO_2, temperature and water availability on above- and belowground biodiversity, AMF-plant associations and C cycle,
(4) Evaluation of the ecological significance of AMF-plant interactions on above- and below-ground biodiversity and C balance,
(5) Modeling of the C-balance processes in grassland ecosystems.

TASK 1. Evaluation of AMF biodiversity in selected Belgian grasslands

Following the identification of representative Belgian grasslands, an assessment of the AMF biodiversity was performed from soils collected at five different sites in the Flemish and Walloon regions. Analyses showed the presence of AMF spores in each soil sample of the different sites. Twenty to fifty spores were recovered per 100 g of air-dried soil. Morphological observations revealed the presence of several strains/species of AMF at each of the five sites. Trap cultures of bulk soil were established on *Allium porrum* L (leek) and maintained under greenhouse conditions to produce AMF material (i.e. spores and intraradical fungal structures). Spores of the same morphotype were subsequently isolated and re-established on leek trap plants to produce monospecific species used for species identification and germplasm preservation. Morphological characteristics of the spores allowed identifying the AMF at the genus level. All the strains collected belonged to the *Glomeraceae* and *Diversisporaceae* families.

Further taxonomic and phylogenic analyses will be performed on the AMF beyond the time frame of the project using morphological and molecular tools. Finally, long-term preservation of the *in vitro* cultures will be initiated. These cultures will be deposited into the Glomeromycota IN vitro COllection (GINCO).

TASK 2. Role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands and their feedbacks on AMF

AMF are able to interconnect different plants through a common mycorrhizal network (CMN). It favors the transport of soil-derived nutrients (e.g. phosphorus) and plant-derived carbon within the network and possibly between plants. It was observed that seedlings established more easily within an existing mycorrhizal network, most likely because they have a rapid and direct access to a large pool of soil nutrients through the mycorrhizal network or even directly from other plants. Moreover, because the mycorrhizal network is already connected to the surrounding vegetation, seedlings could

access carbon from the established plants through this CMN. This could thus imply that the carbon cost of the seedling to the mycorrhizal network is strongly reduced or even inexistent, therefore favoring the seedling establishment. It can also be hypothesized that in a plan community, carbon could circulate from plants to plants through a CMN depending on source/sink relationships.

The transfer of carbon through a CMN, from a *Medicago truncatula* Gaertn. donor plant to a receiver plant, was investigated under highly controlled *in vitro* conditions. Two scenarios were considered:

- The receiver plant was growing under decreased light conditions,
- The receiver plant was a seedling.

Following labeling of the donor plant with $^{13}CO_2$, ^{13}C was detected in the donor plant shoots and roots, in the extraradical mycelium and in the receiver plant roots. Fatty acid analysis of the receiver's roots showed a ^{13}C enrichment in the AMF-specific lipids, while no significant ^{13}C enrichment was detected in the plant-specific lipids.

We concluded that:

(1) Carbon could be transferred from a donor to a receiver plant via a CMN, but remained within the intraradical AMF structures of the receiver's roots and was not transferred to the receiver's plant tissues.
(2) CMN do not sustain seedling establishment through the transfer of carbon,

TASK 3. Impact of elevated CO_2, temperature and water availability on above- and belowground biodiversity, AMF-plant associations and C cycle.

Due to their key position at the soil-root interface, it is of critical importance to include AMF in studies on the impact of global change on plant communities. Consequently, any climate change affecting plants is likely to affect AMF and plant-AMF interactions. Elevated temperatures or changes in precipitation rates may have direct impact on both the AMF and their host plants, while elevated CO_2 can directly impact plant C fixation and indirectly AMF by an alteration of below-ground C allocation. AMF are known to contribute to the C sequestration into the soil and therefore it is essential to elucidate their role in the C cycle under changing climate.

Two microcosm experiments conducted under *in vitro* conditions were designed to investigate the effect of elevated CO_2 and temperature on:

- AMF growth,
- N and P transport by AMF.

In vitro culture systems were placed in either ambient (22/18°C day/night and 380 ± 15 ppm CO_2) or elevated CO_2 (eCO_2: 600 ± 15 ppm CO_2) and temperature (e°T: ambient +3°C) conditions (same photoperiod 16/8h and relative humidity of 70%). Shoot length, number of leaves, root length, root and shoot fresh and dry weight, number of spores, extraradical hyphae length and intraradical root colonization were estimated.

A short-term dynamic (16 days) study of root colonization of *M. truncatula* plantlets and a long-term dynamic (8 weeks) study of spore production and extraradical mycelium development from *M. truncatula* plantlets grown in a mycelium network were conducted. For the short-term dynamic study, the plants grew significantly as shown by all plant parameters measured, except for the shoot length. No differences were observed between plants exposed to either ambient or eCO_2 and e°T conditions at the different time of observations (i.e. 4, 8, 12 and 16 days after the introduction of the plantlets into the mycelium network), except at day 16 where shoot dry weight of plants exposed to elevated CO_2 and temperature was significantly higher as compared to the plants in the ambient conditions. The number of spores and hyphal length did not differ at any date between the two climate conditions, except at day 8 where the number of spores was higher in the systems exposed to elevated CO_2 concentration and temperature as compared to the systems exposed to ambient conditions. Concerning the root colonisation, no difference was observed at any date between the two climate conditions. For

the long-term dynamic study, results on spore production and extraradical hyphae length showed no clear tendency between the two climatic conditions. However, at week 2, 6 and 8, hyphal length in the systems exposed to elevated CO_2 and temperature conditions was higher as compared to the systems exposed to ambient conditions.

hyphale était plus élevée aux semaines 2, 6 et 8 de cette expérience lorsque les cultures se trouvaient en condition de CO_2 et de température élevées.

These results tended to indicate that eCO_2 and e°T could influence:

(1) AMF soil exploration and resources foraging,

The fact that spores production was not affected by eCO_2 and e°T, but that hyphal length increased, showed that the capacity of AMF to explore and exploit new environments as well as colonize new plants could be higher due to:

(1) Increased dispersion of spores,
(2) Higher probability of colonization of surrounding plants.

Three mesocosms studies were conducted.

Study 1 investigated:

(1) How climatic change and the presence of AMF affected grassland communities' CO_2 fluxes, above- and belowground biomass, and leaf nitrogen (N) and phosphorus (P) relations,
(2) Whether interaction effects of climatic factors and presence of AMF were playing a significant role on CO_2 fluxes, biomass and leaf nutrient content in the AMF-plant system.

Study 2 was performed on a soil collected from a Belgian grassland field taking advantage of the richness of AMF that the soil contained. The main objective of this study was to investigate the effect of climate change on AMF root colonization, communities' CO_2 fluxes, biomass and above-ground N and P relations.

Study 3 was conducted to determine the influence of an AMF community on carbon and nitrogen allocation productivity and community structure of grassland communities, both under the current climate and a future climate scenario.

For these studies, growth chambers were exposed to ambient air temperature (T_{air}) and 380 ppm of CO_2 (Amb), while others were continuously warmed at 3ºC above T_{air} and exposed to 610 ppm CO_2 concentration (future climate, $T+CO_2$). Plant communities were assembled using six perennial species, chosen for their co-occurrence in Belgian grasslands, selected from three functional groups that were equally represented in each community: two grasses (*Poa pratensis* L., *Lolium perenne* L.), two N-fixing dicots (*Medicago lupulina* L., *Lotus corniculatus* L.), and two non-N-fixing dicots (*Rumex acetosa* L., *Plantago lanceolata* L.).

For **study 1,** the soil was pasteurized with two 8-hours cycles at 90 °C and communities were planted in soil that was either (i) pasteurized (non mycorrhizal – NM) or (ii) pasteurized and subsequently inoculated with AMF (AMF). Each inoculated community received 100 g of inoculum containing two AMF taxa, *Gigaspora margarita* and *Glomus intraradices*.

For study 2, communities were established in containers filled with un-manipulated soil (natural soil, non-treated with pasteurization) collected from the grassland field.

In **study 3** pasteurized or pasteurized and subsequently inoculated soil was used. The inoculum contained two AMF taxa: *Glomus intraradices* and *Glomus fasciculatum*. At three different times throughout the growing season ecosystems were labeled with ^{13}C and ^{15}N. In this way more detailed information on C and N allocation in AMF and the different plant compartments could be obtained.

Conclusions for **study 1**:

(1) CO_2 fluxes of newly established grassland communities were not significantly affected by AMF in the two climate scenarios considered, although AMF root colonization was higher under combined increased temperatures and CO_2 concentrations,
(2) Under the future climate scenario gross primary productivity (GPP) was slightly higher, determining increasing root biomass only in the absence of AMF suggesting different strategies of below-ground C allocation in the presence of AMF,
(3) On the long term the probable allocation of C to the AMF pool instead of to the root biomass observed in this study could affect below-ground processes such as respiration and, consequently, grassland community CO_2 fluxes.

Conclusions for **study 2**:

(1) Newly established grasslands could benefit from the future climate scenario in terms of total growth,
(2) Carbon was allocated mainly to above-ground plant parts, whereas roots and AMF did not increase under the future climate scenario. Together with the increased soil respiration (R_{soil}) in $T+CO_2$ as compared to *Amb*, this points towards a negative effect on soil C sequestration.

Conclusions for **study 3**:

The results of this study are currently processed. Only above-ground biomass has been analyzed so far.

(1) Above-ground biomass was positively influenced by the future climate scenario,
(2) At the end of the growing season AMF had a positive influence on the above-ground biomass under both climate scenarios.

Further conclusions will be formulated beyond the time frame of the project.

TASK 4. Evaluation of the ecological significance of AMF-plant interactions on above- and below-ground biodiversity and C balance.

This task was planned for the second phase of the project.

TASK 5. Modeling of the C-balance processes in grassland ecosystems

The ANAFORE model (ANAlysis of FORest Ecosystems) was modified to simulate grassland ecosystems in order to evaluate the impact of climate and AMF on the C cycle. ANAFORE includes the effects of additional factors affecting growth such as elevated atmospheric CO_2, fertilization, drought, ozone, and temperature extremes. The data collected during 2007 and 2008 will be implemented into the ANAFORE model in 2009.

1. INTRODUCTION

1.1 Context

Following the report from the SBSTTA and IPCC emphasizing the linkages between biodiversity and climate change (including increasing CO_2 concentration) and their overall relevance in the implementation of sustainable development, the international research effort is now oriented to provide the decision-makers with knowledge and tools to comprehend these interlinkages with the objectives to preserve biodiversity, maintain ecosystem processes and properties and mitigate climate change. In this respect, improved management of grasslands has been identified by the SBSTTA and IPCC as a potential tool to mitigate climatic change by enhancing carbon (C) storage in soils and vegetation, while conserving biodiversity.
Historically the above-ground and below-ground components of ecosystems have been considered separately. However, several recent studies have revealed the influence of both these components on each other. These studies also have demonstrated the fundamental role played by above- and below-ground feedbacks in controlling community and ecosystem processes and properties.
In this respect, arbuscular mycorrhizal fungi (AMF) are of particular interest as they can affect plant community structure, diversity and productivity and be influenced by plant community by feedback responses. Furthermore, AMF constitute a key component contributing to soil C fluxes and to the long-term soil C pools by sequestering up to 15% of the grassland soil organic C.
In MYCARBIO, we focus on Belgian grassland ecosystems and investigate for the first time the role of the symbiotic associations between AMF and plants. In the context of global climate change and impact on biodiversity and ecosystem functioning, this approach is of particular interest as AMF and plant communities are known to (1) influence each other, (2) be involved in several major ecosystem processes, including C cycle, and (3) be affected by climate change.

Objectives: MYCARBIO aims to investigate the impact of AMF on biodiversity and C cycle in Belgian grassland ecosystems under changing climate conditions. To achieve this major objective, five specific objectives have been identified: (1) the evaluation of AMF biodiversity in selected Belgian grasslands, (2) the determination of the role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands, (3) the understanding of the impacts of elevated CO_2, temperature and water availability, on AMF and plant biodiversity, AMF-plant associations and C cycle, (4) the evaluation of the ecological significance of AMF-plant interactions on above- and below-ground biodiversity and C balance and (5) the modelling of the C-balance processes in grassland ecosystems.

1.2 Objectives and expected outcomes

Expected Outcomes: MYCARBIO aims to provide significant insights on the impacts of climate change on grassland ecosystems and biodiversity, which would be valuable for scientists, stakeholders, and policy makers at national and international levels. Among them we identified:

- The first consequent study of the biodiversity of AMF in Belgian grasslands. This will include taxonomic identification, phylogenic classification and germplasm preservation in BCCM^{TM}/MUCL. This study could therefore serve to monitor AMF biodiversity evolution under climate change when future assessment will be undertaken.
- The in-depth investigation of the functioning of grasslands by studying the relationships between above- and below-ground communities. This will provide essential information for ecosystem preservation planning.
- The better comprehension of the impact of climatic change on AMF-plant interactions and their effect on AMF and plant biodiversity and C cycle.
- A first evaluation of the ecological significance of AMF-plant interactions on above- and below-ground biodiversity and C balance in the context of climate change.

- The refinement of the process-based simulation model ANAFORE to provide data to plan and evaluate actions and policies in relation to below- and above-ground biodiversity and C cycle in the context of climate change. In particular, this model could be used to estimate C sinks in relation to article 3.4 of the Kyoto Protocol to report and monitor changes in soil C stocks necessary to provide sufficient level of "verifiability" required under this article.

2. CONFORMITY WITH THE INITIAL PLANNING

2.1 Workplan

WP 1 - Assessment of the AMF biodiversity in selected Belgian grasslands

Following identification of representative Belgian grasslands, soil was collected at 5 different sites. The first site is situated in the Flemish region, while the other four sites are in the Walloon region. Preliminary observations showed the presence of AMF spores in each soil samples of the different sites. Trap cultures on leek were subsequently initiated to produce fungal material (i.e. spores and intraradical fungal structures) and for germplasm preservation. This material is being used for taxonomic and phylogenic identification of the AMF species present in the sites. Further taxonomic and phylogenic analyses will be performed on the AMF beyond the time frame of the project using morphological and molecular tools. Finally, *in vitro* cultures of the AMF produced under greenhouse conditions will be initiated for long-term preservation. These cultures will be deposited into the Glomeromycota IN vitro COllection (GINCO).

In year 1, work was completed for task 1.1: Identification of representative Belgian grasslands**.** Task 1.2: Taxonomic identification, phylogenic classification and germplasm preservation of the AMF collected **was started but will be continued beyond the time frame of the project (12 man/months).** Task 1.3: Valorisation **will be done beyond the time frame of the project.**

WP 2 - Role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands and their feedbacks on AMF

- Three microcosm experiments were performed, in order to study the transfer of C between plants linked by an AMF network of extraradical hyphae. In these experiments, an established plant of *Medicago truncatula* Gaertn. was used as C-donor plant and three different C-receiver plants were considered: (1) a *M. truncatula* seedling, (2) a *M. truncatula* established plant, developing in shadow-conditions and (3) a seedling of *Centaurium erythraea* Rafn., which is a partially myco-heterotrophic plant.
- One mesocosm experiment was conducted during summer 2008 in order to study the effect of the presence of the AMF network on seedling establishment. Seedlings were grown inside a central core with a mesh bag and were surrounded by an established grassland community. The mesh bag allowed (a) both roots and AMF (1 mm pores) to proliferate in the central core, (b) excluded only roots (25 μm pores) or (c) excluded both roots and AMF (1 μm pores). One year after the establishment of the experiment, the comparison between the treatments failed due to the heavy AMF contamination in the central core of the containers where seedling were suppose to grow in the absence of AMF.

In year 1, work was performed on **task 2.1: Identification of the role of AMF for seedling establishment of native and invasive grassland species**, in microcosm (task 2.1.1) and mesocosm (task 2.1.2) experiments. Task 2.1.1 was completed. Task 2.1.2, experiments failed. **Task 2.2: Determination of the role of AMF on plant community structure, diversity and productivity in grasslands and vice versa**. Task 2.2.1 was completed. **Task 2.3: Valorization**: Results obtained in this WP were valorized by 2 master theses (Goubau, 2007; Simon, 2007) and a scientific article published in FEMS Microbiology and Ecology by Voets et al. (2008). The results of the experiments performed in WP2 were presented during the progress meetings, held in year 1 and year 2.

WP 3 - Impact of elevated CO_2, temperature and water availability above- and below-ground biodiversity, AMF-plant associations and C cycle

- Two microcosm experiments were designed to investigate the effect of elevated CO_2 and temperature on (1) the growth of AMF and (2) on the transport of N and P by AMF, under *in vitro* conditions. Results of the experiment 1 were obtained in June 2008. Experiments 2 is

still running and are expected to be fully collected and analyzed beyond the time frame of the project.

- During 2007 a mesocosm experiment was performed in sunlit climate-controlled chambers in order to evaluate the role of AMF on the C-balance, biodiversity and AMF-plant interactions under changing climate. The different climate treatments consisted in (i) ambient temperature (T_{air}) and atmospheric CO_2 concentration (380 ppm) and (ii) T_{air} + 3ºC and elevated CO_2 concentration (610 ppm). Plant communities were grown in soil that was either (a) pasteurized, (b) pasteurized and subsequently inoculated with two different AMF taxa and (c) untreated. The six plant species, selected for their natural co-occurrence in Belgian grasslands, belonged to three functional groups, equally represented in each community: two grass species (*Lolium perenne* L. and *Poa pratensis* L.), two N-fixing dicots (*Lotus corniculatus* L. and *Medicago lupulina* L.), and two non N-fixing dicots (*Plantago lanceolata* L. and *Rumex acetosa* L). Each community contained 18 individuals (three individuals per species). Total ecosystem C fluxes (net ecosystem exchange of CO_2, total ecosystem respiration, and soil respiration) were evaluated three times during the summer. Following each C flux measurement period, a set of ecosystems was harvested and above-ground biomass of individual species as well as below-ground biomass of the whole ecosystem was determined. In addition ecosystems were harvested at the end of October. Other measurements at the plant scale collected at the time of ecosystem harvest included species survival, total leaf area and specific leaf area.

With these experiments, part of **Task 3.1 – Experimentation** was fulfilled. Both the microcosm (task 3.1.1) and the mesocosm (task 3.1.2) experiments were planned starting from month 1 till the end of phase 1. Since this WP has to be completed in the middle of year 3, experiments in this WP will be fully collected and analyzed beyond the time frame of the project. **Task 3.2 – Valorisation**: preliminary results were presented during the meetings, held in year 1 and year 2 and in a scientific article submitted to Mycorrhiza by Voets et al. (2008) (Annex 2.1). Results obtained in the two mesocosm experiments were presented to the follow-up committee and three manuscripts were submitted to peer-review journals. These results will also be integrated into the grassland community C-balance model developed in WP5.

WP 4 - Evaluation of the ecological significance of AMF-plant interactions on above- and below-ground biodiversity and C balance.

This task was planned for the second phase of the project.

WP 5- Modeling of the processes describing C balance in grassland ecosystems

- The ANAFORE model (ANAlysis of FORest Ecosystems) was modified to simulate a grassland ecosystem in order to evaluate the impact of climate and AMF on the C cycle. ANAFORE includes the effects of additional factors affecting growth such as elevated atmospheric CO_2, fertilization, drought, ozone, and temperature extremes. The data collected during 2007 and 2008 will be implemented into the ANAFORE model in 2009.

The task 5.1- Modeling is underway. The first data are being implemented in the model. This task was planned starting from month 7 and was continued in year 2. **Task 5.2 – Valorisation**: preliminary results were presented at the meetings, held in year 1 and in year 2 and also in published paper by Deckmyn et al. (2008) (Annex 2.2).

3. COLLABORATION AND SYNERGY WITHIN THE NETWORK

- Mecosoms experiments were set-up jointly. The UCL team was responsible for the pasteurization of the soil (during year 1) as well as the selection and inoculation of the AMF taxa maintained at UCL (year 1 and 2). The UA partners were responsible for the plant and ecosystems establishment and the experiment measurements. At the time of ecosystems harvest (4 periods during 2007 and 2008) the two teams worked jointly for sampling and measuring of the below-ground component. Soil and plant analyses were performed by UA while AMF analysis was conducted at UCL. This very close collaboration resulted in three joint publications submitted for publication (See point 8)
- Frequent exchanges of information and short visits to UA or UCL were conducted at regular intervals for the follow-up of the experiments.
- A collaboration for the evaluation of the ^{13}C signature of neutral fatty acids in AMF was established with Prof. Pål Axel Olsson (Lund University, Sweden). This will possibly generate a manuscript with co-authorship with the team from the Ecology group of the Lund University.
- A collaboration with Prof. Mathias Rillig from Freie Universitaet Berlin was established in order to evaluate the hyphal length and soil aggregates. The results will be part of a manuscript based on experiments performed in WP 3 between UCL and UA.

UCL and UA have started the writing of a common EU Marie Curie project on the impact of global change on AMF. This project (if successful) will complement the MYCARBIO project by extending the partnership to other EU laboratories.

4. IMPLEMENTATION OF THE METHODOLOGY

The general methodology to address the objective of MYCARBIO was based on a research at different scales in which the level of complexity increases from individual species to community and from specific mechanisms to ecosystems functioning. From this approach and in accordance with the objective of MYCARBIO, five work-packages (WP) were identified:

WP 1 - Assessment of the AMF biodiversity in selected Belgian grasslands

The scientific methodology adopted to assess the AMF biodiversity in Belgian grassland followed this scheme:

1. Identification of representative Belgian grasslands
2. Sampling of roots and soils in selected grasslands
3. Taxonomic identification, phylogenic classification and germplasm preservation of the AMF collected

1. Identification of representative Belgian grasslands

It was decided, in agreement with the follow-up committee, to select 5 different sites. The first site is situated in the Flemish region, while the four other sites are in the Walloon region. The site in the Flemish region was selected by Prof. I. Nijs (UA) and will be used in all mesocosm experiments conducted at the Drie Eiken experimental Platform (UA). The sites in the Walloon region were selected following advice of Prof. B. Toussaint (Unité d'écologie des prairies et des grandes cultures, UCL) and Dr. R. Lambert (Centre agro-environnemental de Michamps, UCL).

2. Sampling of roots and soils in selected grasslands

The roots and soil were collected using a soil probe at the different sites. In order to obtain appropriate samples (or primary samples), five sub-samples (or secondary samples) were taken along a diagonal transect (Annex 1.1). The soil probe allowed for taking soil cores of ± 5 cm Ø and up to a depth of ± 25 cm.

The soil samples were collected in April 2007 for the Flemish site and in August 2007 in the Walloon sites. The soil samples were subsequently stored in the dark at 4°C.

3. Identification

3.1 Isolation of spores from soils

Spores of AMF were collected by wet sieving (Gerdemann and Nicolson, 1963) on a column of 3 successive sieves (500 µm, 106 µm and 38 µm mesh sizes).

The sieving of each mesh was then collected and placed in a 50 ml centrifugation tube in which 25 ml of 50% sucrose (w/v) was added (i.e. sucrose density centrifugation technique (Daniels and Skipper, 1982). The tubes were then placed in a centrifuge (2000 rpm for 5min.) to separate the spores from the soil particles. The spores were then picked with a micropipette, rinsed in water and placed in a Petri plate for morphotype selection.

3.2 Morphological observation of spores for identification

AMF identification is mostly based on the morphology of spores because extraradical hyphae and structures such as arbuscules and vesicles are not species-specific. Spores collected from soil are often damaged and are most often used for identification at the genus level. For identification at the species level, the cultivation of AMF on a trap plant is required. After a couple of months, newly produced spores were sampled from the pot cultures and used for identification (see point 3.3 in this section). At least 30-50 spores from the same morphological type are necessary.

3.3. Culturing AMF

Trap cultivation on a suitable host plant is necessary to obtain many healthy spores used for identification and as inoculum to establish mono-species cultures. Spores collected directly from a field soil may be inadequate for several reasons: (a) they may appear healthy but are not viable (some

persisting as dead husks for years or possibly decades), (b) they may loose or change appearance of their structural characters in response to root pigments, soil chemistry, temperature, moisture, and microbial activity, and (c) they may represent only those colonizing AMF with enough activity and biomass to trigger sporulation. These constraints are less marked with trap cultures
Trap cultured are preferably initiated by using:

- Soil and mycorrhizal root pieces
- Isolated spore morphotypes
- Monospores

In this project, we used collected soil and mycorrhizal root pieces and isolated spores to initiate trap cultures. The substrate used was a mix of Terragreen and sand (2:1) and the host plant used was *Allium porrum* L. The pots were placed in Sunbags (Sigma Co.) in order to avoid cross-contamination (Walker and Vestberg 1994). The cultures were initiated in August 2007 and are currently maintained in greenhouses at UCL until sufficient spores are produced to perform taxonomic and phylogenic characterization, but also to initiate mono-species cultures. Every month since October 2007, a sample of substrate from each pot was observed to follow the development of the AMF.

3.4. Germplasm preservation

Germplasm preservation can be achieved in pot cultures but also under *in vitro* conditions (Annex 1.2). In MYCARBIO, the AMF were cultured mono-specifically using both options.

1- Identification of representative Belgian grasslands

- Site 1: Sandy-loam soil, collected from relatively species-rich, extensively managed grassland in Berlaar (province of Antwerp). Sampled in April 2007 and again in August 2007.
- Site 2 & 3: Temporary (2 years) & permanent (< 20 years) grasslands
- Site 4 & 5: Temporary (2 years) grasslands with or without N fertilisation & OM amendments

sampled in August 2007. These sites are situated in the experimental station of Michamps (UCL).

2- Sampling of roots and soils in selected grasslands

Soil characterisation was performed by the Bodemkundige Dienst van Belgie vzw (Heverlee) for the site 1and the Unité des Sciences du Sol of the Université catholique de Louvain (Louvain-la-Neuve) for sites 2 to 5 (Data not shown).

Vegetation characterisation (i.e. for the preponderant species present) need to be done at site 1 and was done by Dr. R. Lambert at the site 2, 3, 4 and 5:
Site 2: *Lolium perenne* L., *Dactylis glomerata* L., *Trifolium pratense* L.
Site 3: *Lolium perenne* L., *Poa trivialis* L., *Dactylis glomerata* L.
Site 4: *Lolium perenne* L., *Elytrigia repens* L.
Site 5: *Lolium perenne* L., *Trifolium repens* L.

3- Morphological observation of spores for identification

Early observations from the soil collected at the five different sites showed that 20-50 spores could be recovered per 100 g of air-dried soil. Morphological observations revealed that several strains/species of AMF were present are each of the 5 sites. However, due to the low number of spores and the poor quality of the fungal material (i.e. spores in bad health conditions), further identification was not possible to achieve. Nonetheless, spores isolated were grouped by type in order to start monospecific pot cultures of AMF. The analysis performed (June 2008) on the monospecific-AMF pot cultures showed that four strains were successfully reproduced under greenhouse conditions and morphological description was conducted at genus level (Annexes 1.3, 1.4, 1.5 and 1.6). Further taxonomic and phylogenetic analyses of the spores will be performed at the end of year 2. Sequences will be deposited in the international GenBank.

Culturing AM fungi

The cultures were initiated in August 2007 and are currently maintained in greenhouses at UCL until sufficient spores are produced to perform taxonomic, phylogenetic characterization. New monospecific-AMF pot cultures were initiated in June 2008 and morphological identification were performed in October 2008.

Germplasm preservation.

Currently, AMF species are proliferating on trap cultures. This represents the first step towards their preservation. When enough spores and high root colonization will be obtained, the AMF will be cultured under *in vitro* on root organ cultures and autotrophic *Medicago truncatula* plantlets.
Further taxonomic and phylogenic analyses will be performed on the AMF beyond the time frame of the project using morphological and molecular tools. Finally, long-term preservation of the *in vitro* cultures will be initiated. These cultures will be deposited into the Glomeromycota IN vitro COllection (GINCO).

WP 2 - Role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands and their feedbacks on AMF

In this WP, three microcosm studies were performed and one mesocosm experiments was started.
In the microcosm experiments, the possible flow of C from a donor to a receiver plant via the AM fungal network was investigated under *in vitro* conditions. Three experiments were performed with *Medicago truncatula* as C-donor plant. The receiver plan was either a *M. truncatula* seedling (Exp 1.), a *M. truncatula* established plant, developing in shadow-conditions (Exp 2.) and a seedling of *Centaurium erythraea*, which is a partially myco-heterotrophic plant (Exp 3.)

(1) The first experiment was designed with an established *M. truncatula* plant as a C-donor and a *M. truncatula* seedling as a C-receiver. In a bi-compartmental Petri plate (diameter 90 mm), a *M. truncatula* Cv. Jemalong J5 was plated on the Modified Strullu Romand medium (Declerck et al., 1998; modified from Strullu and Romand, 1986) without sugar and without vitamins, solidified with 3 g l^{-1} Gelgro^{TM} following the method described in Voets et al. (2005) (the donor compartment). The roots of the plant were inoculated with spores of the AMF *Glomus intraradices*. When the plant was colonised by the AM fungal hyphae and the hyphae crossed the partition wall (5-6 weeks after the inoculation of the spores), two 4-days old plantlets were inserted in the second compartment (the receiver compartment), in contact with the established network of AMF hyphae: a *M. truncatula* plant J5 and a *M. truncatula* plant TRV25, which is a non-mycotrophic clone of the J5 plant, that served as a control plant. Nine days after the insertion of the two plantlets, the adult donor plant J5 was labelled with $^{13}CO_2$. After a chase period of 6 days, the ^{13}C was measured in the different structures of the three plants and in the extraradical fungal network.

(2) The second experiment was designed to investigate whether C was transferred from an established plant, developing in full light conditions, to a same established plant, developing in shadow-conditions. The system used in this experiment was identical to the system described in experiment one, except that the three plants were inserted in the Petri plates at the same time (For details see annex 1.7). As in experiment one, only the donor plant was inoculated with AM fungal spores. At week 12, when a profuse network of extraradical hyphae was established in the Petri plates, on both sides of the partition wall, linking the donor plant (J5-D) in the donor compartment (DC) and the receiver plant (J5-R) in the receiver compartment (RC), the donor plants were labelled with $^{13}CO_2$. In order to create a sink for C, three days before labelling and until the end of the chase period (6 days), the light intensity above the plants in the receiver compartment (the receiver plant and the control plant) was reduced to half the initial light intensity, without affecting the light intensity above the donor plants.

(3) Since no C-flow was detected between two *M. truncatula* plants, a third experiment was designed in which a different combination of plants was used in order to investigate if a C-flow between plants is possible under some extreme conditions. The C-flow was studied from an established *M. truncatula* plant to a seedling of *C. erythraea*, a partially myco-heterotrophic plant that, in its seedling phase, seems dependent of AMF hyphae and surrounding plants for its C supply (Grime et al., 1987). The two plants (*M. truncatula* and *C. erythraea*) were established in two separated compartments of a bi-compartmented Petri plate and were connected by a mycelial network of *G. intraradices*, in an identical way as described in the experiments above. *M. truncatula* was labelled by one pulse of ^{13}C-CO_2 and the transfer of C from *M. truncatula* to *C. erythraea* was measured. *C. erythraea* was growing in a plastic tube, connected to the Petri plate following the methods described in Dupré de Boulois et al. (2006) and in annex 1.8. For this experiment, a non-mycorrhizal control treatment was also included. This treatment was identical to the treatment described above, except that no mycorrhizae were inoculated in these plates.

- First experiment: Identification of the role of AMF for seedling establishment of native grassland species

In the first experiment, the C-flow was measured from an established *M. truncatula* plant to a *M. truncatula* seedling. After labelling with $^{13}CO_2$ and a chase period of 6 days, the ^{13}C label was recovered from the different structures (leaves, stem, roots) of the donor plant and in the extraradical fungal network. No ^{13}C was recovered from the stem and the leaves of the plants in the receiver compartment (the receiver plant and the control plant) (Table 1). The roots of the two plantlets in the receiver compartment were not entirely subjected to ^{13}C analysis but, in order to determine the exact location of the ^{13}C in the roots, the roots of these plantlets were subjected to a fatty acid extraction. The results showed that ^{13}C was retrieved in the lipids inherent to the AMF (16:1ω5), but only very little ^{13}C was retrieved in the lipids of the plant roots (18:2ω6.9), and this in the same quantity in the roots of the receiver plant as in the roots of the control plants (Table 1). This indicates that the ^{13}C, assimilated by the donor plant, was transferred to its roots and to the associated AMF but that the ^{13}C was not transferred to the root cells of the seedling. In this situation, the establishment of seedlings seems thus not dependent from a C-flow from the already established plants growing in the direct neighbourhood of the seedlings.

Table 1: Delta ^{13}C values (‰) in the different fractions of the plants and the fungus in the culture system in experiment 2 (Sink: Seedlings) (J5-D: *Medicago truncatula* (line J5) – donor plant; J5-R: *Medicago truncatula* (line J5) – receiver plant; TRV25: *M. truncatula* (line TRV 25); ERM: extraradical mycelium of *Glomus intraradices*), together with the background $\delta^{13}C$ values in the tissues. NLFA: Neutral Lipid Fatty Acids. The NLFA 16:1ω5 represents the main neutral lipid fraction in the AM fungus, NLFA 18:2ω6.9 is present in plant storage lipids (and in a little quantity in the AM fungus). Data of the seedlings are means of 3 replicates ± SE. The background values are means of 5 replicates ± SE, except the background $\delta^{13}C$ value of NLFA 16:1ω5 (n=1).

	J5-D plant	J5-D roots	ERM†	J5-R roots		J5-R stem	J5-R leaves	TRV25 roots		TRV25 stem	TRV25 leaves
				NLFA 16:1w5	NLFA 18:2w6.9			NLFA 16:1w5	NLFA 18:2w6.9		
Sink: Seedlings	848.5 ± 178.0	42.7 ± 37.0	67.3 ± 21.0	37.0 ± 27.1	-19.5 ± 8.0	-31.6 ± 0.9	-34.4 ± 1.2	25.6 ± 36.6	-21.1 ± 6.9	-29.7 ± 2.2	-34.9 ± 1.2
Background $\delta^{13}C$ value	-34.6 ± 0.3	-31.3 ± 0.4	-35.0 ± 0.2	-42.7	-29.8 ± 0.3*	-33.2 ± 0.3	-35.0 ± 0.4	-42.7	-29.6 ± 0.2*	-30.6 ± 0.6	-34.1 ± 0.8

* The background $\delta^{13}C$ value of the NLFA 18:2ω6.9 for both roots (J5-R and TRV25) could not be measured. Therefore, the presented background values are the natural $\delta^{13}C$ values of a non-mycorrhized non-labeled root of the corresponding plant.

†The presented $\delta^{13}C$ value is the mean value from both the DC and the RC.

The detailed experiment is described in:

- Goubau, I. 2007. Rôle de champignons mycorhiziens à arbuscules dans le transfert de carbone d'une plante adulte autotrophe à une jeune plantule de la même espèce. Mémoire présenté à l'Université catholique de Louvain.
- Voets et al. (2008) FEMS Microbiology and Ecology 65: 350-360 (See annex 2.3).

- Second experiment: Identification of the role of AMF on plant community structure and species richness level in grassland and vice versa.

In order to determinate the role of AMF on plant community structure, an experiment was designed to investigate whether C was transferred from an established plant, developing in full light conditions, to a same established plant, developing in shadow-conditions. This experiment was performed with plants of *M. truncatula* cv. Jemalong, under *in vitro* conditions. Three plantlets of *M. truncatula* were inserted in a bi-compartmental Petri plate (90 mm diameter), following the methods described in Voets et al., 2005 and following the design of Figure 3. The plantlets were inoculated with AM fungal spores of *Glomus intraradices*. Three weeks after the association of the AM fungal spores to the roots of *M. truncatula*, the AMF started to colonize the plant and an extensive network of hyphae and spores developed in the Petri plates. After 5-6 weeks, the hyphae started to cross the partition wall and an extensive network of hyphae developed in the second compartment (i.e. the receiver compartment). At week 12, when a profuse network of extraradical hyphae was established in the Petri plates, on both sides of the partition wall, linking the donor plant (J5-D) in the donor compartment and the receiver plant (J5-R) in the receiver compartment, the donor plants were labelled with $^{13}CO_2$. In order to create a sink for C, three days before labelling and until the end of the chase period (6 days), the light intensity above the plants in the receiver compartment (the receiver plant and the control plant) was reduced to half the initial light intensity, without affecting the light intensity above the donor plants.

After the chase period, all the tissues of the three plants (donor (J5-D), receiver (J5-R) and control (TRV25) plants) were analysed with Mass Spectrometry in order to detect the ^{13}C in the respective tissues. Six days after labelling of the J5-D plants with $^{13}CO_2$, a large quantity of ^{13}C was incorporated in the J5-D plant tissues (Table 2). The label was transferred to the roots of the plant and to the extraradical mycelium (ERM), which extended from the DC into the RC. The roots of J5-R showed only a slight increase in ^{13}C, not significantly higher than the ^{13}C enrichment in the roots of the non-mycotrophic control plant (Table 2). In the stem and leaves of both plants developing in the receiver compartment (i.e. J5-R and TRV25), no detectable increase of $\delta^{13}C$ was observed (Table 2).

Table 2: Delta ^{13}C values (‰) in the different fractions of the plants and the fungus in the culture system in experiment 1 (Sink: Established plants) (J5-D: *Medicago truncatula* (line J5) – donor plant; J5-R: *Medicago truncatula* (line J5) – receiver plant; TRV25: *M. truncatula* (line TRV 25); ERM: extraradical mycelium of *Glomus intraradices*), together with the background $\delta^{13}C$ values in the tissues. Values are means of 5 replicates ± SE.

	J5-D plant	J5-D roots	ERM†	J5-R roots	J5-R stem	J5-R leaves	TRV25 roots	TRV25 stem	TRV25 leaves
Sink: Established plants	506.0 ± 78.2	159.8 ± 36.8	152.3 ± 65.3	-29.5 ± 0.9	-33.4 ± 0.3	-34.6 ± 1.5	-28.8 ± 0.9	-30.0 ± 0.4	-34.0 ± 0.5
Background $\delta^{13}C$ value*	-34.6 ± 0.3	-31.3 ± 0.4	-35.0 ± 0.2	-31.3 ± 0.4	-33.2 ± 0.3	-35.0 ± 0.4	-29.6 ± 0.2	-30.6 ± 0.6	-34.1 ± 0.8

†The presented $\delta^{13}C$ value is the mean value form both the DC and the RC.

*The background $\delta^{13}C$ value in the respective tissues is the natural value, measured in non-labeled plants (or in the non-labeled ERM)

These results indicated that, even when a sink was created by shading the receiver plant, no evidence for the existence of a C-flow from the donor to the receiver plant by means of the AM fungal mycelium was found. These results therefore confirmed previous results, indicating that C transferred from autotrophic donor plants to autotrophic receiver plants remains in the receiver's roots and that a transfer to the shoots is unlikely to occur (Robinson and Fitter, 1999; Zabinski et al., 2002; Pfeffer et al., 2004; Fitter, 2006).

Details of these results are given in:

- Voets, 2007. Role of arbuscular mycorrhizal networks on plant interconnection and carbon transfer. PhD thesis at the Université catholique de Louvain, pp: 220.
- Voets et al. (2008) FEMS Microbiology and Ecology 65: 350-360 (See annex 2.3).

- Third experiment: Identification of the role of AMF on plant community structure and species richness level in grassland and vice versa.

Since no C-flow was detected between two *M. truncatula* plants, a different combination of plants was used in order to investigate if a C-flow between plants was possible under some extreme conditions. In order to do so, the C-flow was studied from an established *M. truncatula* plant to a seedling of *Centaurium erythraea*, a partially myco-heterotrophic plant that, in its seedling phase, seems dependent of AM fungal hyphae and surrounding plants for its C supply (Grime et al., 1987). The experiment was performed under *in vitro* conditions. The two plants (*M. truncatula* and *C. erythraea*) were established in two separated compartments of a bi-compartmented Petri plate and were connected by a mycelial network of *G. intraradices*. *M. truncatula* was labelled by one pulse of ^{13}C-CO_2 and the transfer of C from *M. truncatula* to *C. erythraea* was measured. *C. erythraea* was growing in a plastic tube, connected to the Petri plate, following the methods described in Dupré de Boulois et al. (2006) and in annex 1.8. The experimental design of this experiment is identical to the design of the experiment with a seedling of *M. truncatula* as a receiver plant. For detailed materials and methods description can thus be referred to the article of Voets et al. (2008). For this experiment, a non-mycorrhizal control treatment was also included. This treatment was identical to the treatment described above, except that no mycorrhizae were inoculated in these plates.

Seven days after labelling, the shoot, the roots of *M. truncatula* and *C. erythraea*, as well as the hyphal network were collected and analysed by Mass Spectrometry. The quantity of ^{13}C was measured in the leaves and roots of the donor plant as well as in the fungus in the donor and receiver compartment (Table 3). No trace higher than the background value of ^{13}C was detected in the leaves of the non-mycotrophic control plant in the receiver compartment (Table 3). In the leaves of *C. erythraea*, a great increase of ^{13}C was detected, compared to the background values. However, an equivalent rate of ^{13}C was detected in the non-mycorrhized plants (Table 3).

Table 3: Delta ^{13}C values (‰) in the different fractions of the plants and the fungus in the culture system, together with the background $\delta^{13}C$ values in the tissues.

		Delta ^{13}C background (‰)	Delta ^{13}C labelled (‰)
Donor compartment	M leaves	-36 ± 0,2 (4)	1886,3 ± 149,1 (6)
	M stem	-32,3 ± 0,3 (4)	365,2 ± 43,0 (7)
	M flowers	/	106,5 ± 1,0 (6)
	M fruits	/	115,5 ± 0,8 (3)
	M roots	-33,7 ± 0,2 (4)	224,6 ± 42,0 (7)
	G. intraradices	-35,0 ± 0,2 (3)	18,0 ± 14,0 (5)
Receiver compartment	G. intraradices	-35 ± 0,2 (3)	15,4 ± 12,2 (5)
	TRV 25	-32,3 ± 0,5 (4)	-31,9 ± 0,5 (4)
	C. erythraea	-22,5 ± 0,2 (12)	122,9 ± 47,1 (4)
	Control C. erythraea	-22,5 ± 0,2 (12)	138,8 ± 55,3 (2)

M = *M. truncatula*, TRV 25 = *M. truncatula* non-mycotroph.

Values represent the mean of the samples ± SE. The number between the brackets represents the number of repetitions.

The roots of the plants in the receiver compartment (i.e. the non-mycothrophic *M. truncatula* plant and *C. erythraea*) were subjected to an analysis of fatty acids in the roots. Because of two major problems the results of these analyses could not be interpreted in a optimal way. These problems were: (1) *C. erythraea* contained in its tissues traces of the 16:1ω5, a lipid that is generally present in AM fungi but not in plant tissues; (2) also the non-mycotrophic control plant presented traces of the lipid 16:1ω5, while normally, no mycorrhiza could be present in these plant roots. However, this could be due to little hyphal fragments, sticking on the root surface and thus biasing our results.

With the above-described experiments, the exact pathway of the transfer of C between plants could not be ascertained. The leaves of *C. erythraea* showed an increase in ^{13}C; however, so did the non-mycorrhizal control plants of *C. erythraea*. The non-mycotrophic control plant did not show an increase of ^{13}C in the leaves. However, this plant was inserted in the Petri plate with the roots inside the plate but the stem growing outside the plate (Voets et al., 2005). To be an optimal control for this type of experiment, the non-mycothrophic control plant should thus be grown in a tube, exactly in the same way as the receiver plant *C. erythraea*.

An additional experiment was therefore performed with exactly the same experimental set-up as in the previous experiment, except that this time, the control plant was also inserted in a (separate) tube. After labeling of the donor plant and a period of 6 days chase, the ^{13}C was measured in the different tissues of the receiver and control plants.

After the 6-days chase period, a large quantity of ^{13}C was detected in the plant tissues of the donor plant (Table 4). The label was transferred to the roots of the donor plant and to the extraradical mycelium (ERM), which extended from the DC into the RC. The lipid 16:1ω5 in the roots of the receiver plant (*C. erythraea*) as well as the control plant (TRV25) showed an increase in ^{13}C, and so did the lipid 18:2ω6.9 in both roots (Table 4). Also the leaves of both plants showed an increase in ^{13}C. The presence of ^{13}C in the lipid 16:1ω5 of the control plant is probably due to small hyphal fragments, sticking on the surface of the control roots. This has biased the results. The presence of ^{13}C in the leaves of the receiver plant and also in the leaves of the non-mycorrhizal control plant could be due by a C-flow via the air in the Petri plate, following respiration of the roots and the fungus in the plate. This ^{13}C in the air was probably subsequently re-fixed by the leaves of the receiver as well as the control plant.

Table 4: Delta ^{13}C values (‰) in the different fractions of the plants and the fungus in the culture system, together with the background $\delta^{13}C$ values in the tissues.

		Delta ^{13}C background (‰)	Delta ^{13}C labelled (‰)
Donor compartment	M leaves	-36,0 ± 0,2 (4)	1261,5 ± 92,6 (4)
	M stem	-32,3 ± 0,3 (4)	1186,0 ± 307,2 (4)
	M roots	-33,7 ± 0,2 (4)	428,0 ± 72,3 (4)
	G. intraradices	-35,0 ± 0,2 (3)	279,8 ± 88,7 (4)
Receiver compartment	*G. intraradices*	-35,0 ± 0,2 (3)	401,0 ± 67,1 (4)
	16:1ω5 *C. erythraea*	-35,0 ± 0,2 (3)	384,3 ± 91,0 (4)
	18:2ω6.9 *C. erythraea*	-27,91 (1)	52,0 ± 37,0 (4)
	Leaves *C. erythraea*	-22,5 ± 0,2 (12)	49,8 ± 23,0 (4)
	16:1ω5 TRV25	-35,0 ± 0,2 (3)	381,3 ± 54,8 (4)
	18:2ω6.9 TRV25	-29,6 ± 0,2 (5)	142,0 ± 3,5 (4)
	Leaves TRV 25	-32,3 ± 0,5 (4)	74,3 ± 13,5 (4)

M = *M. truncatula*, TRV 25 = *M. truncatula* non-mycotroph.

Values represent the mean of the samples ± SE. The number between the brackets represents the number of repetitions. 16:1ω5 and 18:2ω6.9 are the analysed fatty acids and are generally designated to the fungus and the plant tissues respectively.

As a conclusion, we could state that the experimental set-up used in these experiments, with the receiver plant growing in a tube attached to the Petri plate (Dupré de Boulois et al., 2006), did not make it possible to demonstrate the transfer of C from *M. truncatula* to *C. erythraea*. Another set-up is necessary to exclude not desired pathways of C between the two plants. These experiments however highlight the complexity of *in vitro* C-studies and the importance of improved study systems to perform detailed studies on this subject.

Details of these experiments are described in the master thesis:

- Simon A. 2007. Rôle des champignons mycorhiziens à arbuscules dans le transfert de carbone d'une plante autotrophe à une plante myco-hétérotrophe. Mémoire présenté à l'Université catholique de Louvain, pp: 65.

In order to evaluate the effects of AMF on seedlings establishment (germination, survival, and growth) in grassland communities, one experiment was started at the end of 2007 under mesocosm conditions. Three grassland species, *Lolium perenne* L., *Plantago lanceolata* L., and *Lotus corniculatus* L. were sown in August and transplanted in October 2007 into plastic containers to establish communities. In each container, all three species at four individuals per species were planted in hexagonal grid at a spacing of 4 cm. In the centre of the container, a 5-cm polyester mesh bag, filled with pasteurized (two 8-hours cycles at 90 °C) soil, was placed. The mesh bag allowed (a) roots and AMF (1 mm pores) (b) excluded only roots (25 μm pores), or (c) excluded both roots and AMF (1 μm pores). Nitrogen fertilizer (50 kg ha^{-1} of nitrogen as NH_4NO_3) was added to every ecosystem in two times during the third and fourth week of July 2008. In the first week of August all ecosystems were cut to 3 cm above ground level. One week later, 15 seeds of the three species used in the communities were placed in the centre core with the objective to identify the role of AMF during the seedlings establishment. Moreover, seeds from a non-mycotrophic grassland species (*Rumex acetosa* L.) were also placed in the centre core in some containers, to compare it with seedlings of mycotrophic species. From the day of sowing germination was checked daily to determine germination percentage and timing for each species. After two weeks all the seedlings were removed except the ones that germinated first in every container. Photosynthetically active radiation (PAR) was measured weekly at the soil surface of the central core and above the canopy from the moment of sowing. In mid-October a comparison between soil samples from the different AMF treatments revealed a heavy AMF contamination in the compartments that were supposed to be AMF-free. The heavy AMF contamination did not allow drawing any conclusion regarding the role of AMF for seedling establishment and therefore the experiment was terminated.

WP 3 - Impact of elevated CO_2, temperature and water availability above- and below-ground biodiversity, AMF-plant associations and C cycle

Two microcosm experiments were designed to investigate the effect of elevated CO_2 and temperature under *in vitro* conditions on (1) the growth of AMF and (2) on the transport of N and P by AMF. To conduct these two experiments, it was necessary that the fungus colonize the roots at the early stage of plant growth, hence a new methodology allowing a fast and heavy colonization of the plantlets was developed based in the early results of Goubau (2007) (see WP 2, experiment 1).

The new *in vitro* mycorrhization method adapted to young plantlets, by using the symbiotic phase of the fungus as inoculum was designed to demonstrate (1) the capacity of an AM extraradical mycelium extending from a donor plant to colonize seedlings and (2) the ability of these colonized seedlings to reproduce the fungal life cycle. Details of this method are given in annex 2.1.

Experiment 1. Effect of eCO_2 and e°T on AMF development.

- Short-term time dynamics study of root colonization of *M. truncatula* plantlets grown in an extraradical mycelium network

Using the same methodology described above, the plantlets were transferred into new HAM-P *in vitro* culture systems and placed in either ambient (22/18°C day/night and 380 ± 15 ppm CO_2) or elevated CO_2 (eCO_2 – 600 ± 15 ppm CO_2) and temperature (e°T – ambient +3°C) conditions (same photoperiod 16/8h and relative humidity of 70%). After 4, 8, 12 and 16 days of growth the *M. truncatula* plantlets were harvested. The intraradical root colonization was estimated after staining following the method described by McGonigle et al. (1990). Shoot length, number of leaves, root length, root and shoot fresh and dry (60°C for 48h) weight, and number of spores were measured. The extraradical hyphae and root length was determined as detailed in Declerck et al. (2003), while spore enumeration followed the methodology described in Declerck et al. (2001).

- Long-term course follow-up of spore production and extraradical mycelium development from *M. truncatula* plantlets grown in a mycelium network

After 12 days of growth in the mycelium network in the HC, the plantlets developing in this compartment were carefully removed and subsequently transferred to mono-compartmental HAM-P *in vitro* culture systems, which contained the same MSR medium lacking sucrose and vitamins as described above. These systems were then either placed in ambient (22/18°C day/night and 380 ± 15 ppm CO_2) or elevated CO_2 (eCO_2 – 600 ± 15 ppm CO_2) and temperature (e°T – ambient +3°C) conditions (same photoperiod 16/8h and relative humidity of 70%). Stem length, number of leaves and root length, spore production and extraradical mycelium development was monitored at week 1, 2, 3, 4, 6 and 8, following the methodology detailed in Voets et al. (2005). The extraradical hyphae and root length was determined as detailed in Declerck et al. (2003), while spore enumeration followed the methodology described in Declerck et al. (2001). At week 8, anastomosis density was measured considering anastomoses within and between hyphae. Plant and AMF were then harvested and intraradical root colonization was estimated after staining following the method describe by McGonigle et al. (1990). Root and shoot fresh and dry (60°C for 48h) weight were also measured.

Experiment 2: Effects of eCO_2 and e°T on the transport of N and P by AMF

After 12 days of growth in the mycelium network in the HC, the plantlets developing in this compartment were carefully removed and subsequently transferred to bi-compartmental HAM-P *in vitro* culture systems, which contained the same MSR medium lacking sucrose and vitamins as described above. The plantlets were contained in a root compartment (RC) where roots and AMF developed, while only the extraradical mycelium of the AMF was allowed to cross the partition wall separating the Petri plate to develop into HC. The medium in the HC's was poured according to the methodology of St-Arnaud et al. (1995). The total volume of medium in the HC's was 10ml

Five weeks thereafter, the extraradical mycelium grew profusely into the RC's and HC's. The HAM-P *in vitro* culture systems were then placed in either ambient (22/18°C day/night and 380 ± 15 ppm CO_2) or elevated CO_2 (eCO_2 – 600 ± 15 ppm CO_2) and temperature (e°T – ambient +3°C) conditions (same photoperiod 16/8h and relative humidity of 70%). After 96h, MSR medium lacking sucrose and vitamins (10 ml), but labelled with filter-sterilized (Acrodisc® Syringe Filters, PALL Corporation Ann Arbor, Mi, USA) ^{33}P and ^{15}N, was poured by in the HC's. The source of ^{33}P was orthophosphate in dilute hydrochloric acid (<0.1M) supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK). The concentration of ^{33}P in the medium was 1395 $Bq.ml^{-1}$. ^{15}N-labelled NH_4^+ (concentration in medium of 2mM) was used, as NH_4Cl. A formaldehyde control treatment was also considered following the description of Dupré de Boulois et al. (2005).

Ninety-six hours following ^{33}P labelling, the total extraradical hyphal length, root length and number of spores in the RC's and HC's were estimated. The extraradical hyphae and root length were determined as detailed in Declerck et al. (2003), while spore count followed the methodology described in Declerck et al. (2001). The number of total and active hyphae (i.e. presenting bi-directional flux of cytoplasm/protoplasm) on the partition wall was measured under dissecting microscope (x40 - Olympus SZ40, Olympus Optical Co., (Europa) Gmbh, Germany).

The shoots of *M. truncatula* were then collected by cutting the shoots at the level of the MSR medium contained in the RC's. The medium in the HC's was sampled and the HC's were rinsed twice with 5 ml distilled water. In the RC's, roots were removed from the solidified MSR^{Cs} medium and cleaned-free from the remaining gel and extraradical mycelium using 10mM citrate buffer (Doner and Bécard, 1991). The MSR medium containing extraradical mycelium and dissolve medium in the citrate buffer was then collected.

Shoot and root fresh and dry (60°C for 48h) weight were measured. Two subsamples of roots were then considered: one for determining AMF colonization and one for ^{33}P and ^{15}N measurements. AMF root colonization was observed under compound microscope (x100 to x400 - Olympus BH2, Olympus Optical Co., (Europa) Gmbh, Germany) following clearing with 10% KOH and staining with 0.2% Trypan blue (Phillips and Hayman, 1970). Root colonization was assessed by the evaluation of frequency (%F) and intensity (%I) of AM fungal colonization (Plenchette and Morel, 1996). Arbuscular (%A), vesicular (%V) and hyphal (%H) colonization was assessed (McGonigle et al., 1990).

Transport of ^{33}P was assessed by measuring ^{33}P activities in the medium contained in the HC's and RC's, in the fungal biomass of these compartments, and in the roots and shoots of *M. truncatula*. Prior to ^{33}P counting, fungal and plant samples were dissolved in HClO4 / HNO3 (1:1) solution, and then cleared using H_2O_2 (30% v/v). Solid MSR medium was dissolve using 10mM citrate buffer and fungal biomass was then dissolved using HClO4 / HNO3 (1:1) solution. Liquid scintillation cocktail (Ultima Gold™, Packard BioScience, Groningen, The Netherlands) was then added to all samples in 10 ml aliquots. Samples were then subjected to ^{33}P counting on a Packard TR2500 Liquid Scintillation Analyser (Packard Instrument Co., Meriden, CT, USA). Separately, ^{15}N measurements were performed using gas chromatography/mass spectrometry (GC/MS) analysis following the description of Govindarajulu et al. (2005).

During 2007 two mesocosms studies were established. **In study 1** we investigated whether the presence of AMF changed the intrinsic responses of grassland communities to the exposure to the future climate. For this purpose we evaluated communities' CO_2 fluxes, above-ground and below-ground biomass, and leaf nitrogen (N) and phosphorus (P) relations. **Study 2** was performed on a soil collected from a Belgian grassland to take advantage of the richness of AMF that the soil contained. In this second study we hypothesized that, under the future climate scenario with elevated temperature and CO_2 (a) gross primary productivity and above- and below-ground biomass will increase, mainly because of the enhanced C supply (b) that AMF colonization will increase and that (c) soil respiration (R_{soil}) will be promoted via enhanced below-ground C allocation.

The experimental platform that included both studies was located at the Campus Drie Eiken, University of Antwerp, Belgium (51º 09′ N, 04º 24′ E, 10 m elevation) and consisted of 10 sunlit, climate-controlled chambers with an interior surface area of 2.25 m^2. Five chambers were exposed to ambient air temperature (T_{air}) and 375 ppm of CO_2 (*Amb*), while the other five were continuously warmed at 3ºC above T_{air} and exposed to 620 ppm of CO_2 concentration (future climate, $T+CO_2$). Each chamber contained 30 grassland communities and the experiment described here utilized a subset of 90 synthesized grassland mesocosms (units of soil + grassland vegetation in containers) randomly distributed over the 10 chambers (Annex 1.9). Communities were established at the beginning of May 2007 by transplanting 5-weeks old seedlings in plastic containers of 24 cm inner diameter and 40 cm height, buried into the soil to mimic soil temperature fluctuations. The containers were filled with soil collected from a relatively species-rich, extensively managed grassland in Berlaar (province of Antwerp, Belgium) and classified as sandy with 89.2% sand, 8.7 % silt, and 2.1% clay, pH = 5.3, 1.5 % total C, 117 mg N and 45 mg P per 100 g of air dry soil. **For study 1** the soil was pasteurized with two 8-hours cycles at 90 °C and communities were planted in soil that was either (i) pasteurized (*NM*) or (ii) pasteurized and subsequently inoculated with AMF (*AM)*. Each inoculated community received 100 g of inoculum containing equal proportions (in weight of prepared inoculum) of two AMF taxa: *Gigaspora margarita* BEG34 (± 2 spores per g of inoculum, kindly provided by BIORIZE) and *Glomus intraradices* MUCL 41833, kindly provided by GINCO (± 40 root fragments per g of

inoculum, 85% frequency of root colonization, Plenchette and Morel, 1996). **For study 2** communities were established in containers filled with un-manipulated soil (natural soil, non treated with pasteurization) collected from the grassland field. In both studies plant communities were assembled using six perennial species, chosen for their co-occurrence in Belgian grasslands, selected from three functional groups that were equally represented in each community: two grass species (*Poa pratensis* L., *Lolium perenne* L.), two N-fixing dicots (*Medicago lupulina* L., *Lotus corniculatus* L.), and two non-N-fixing dicots (*Rumex acetosa* L., *Plantago lanceolata* L.). Each community contained 18 individuals planted in a hexagonal grid with a 4.5 cm interspace between plants. During the duration of the experiment the communities in the two climate scenarios received equal irrigation regimes so that any enhanced water consumption due to the elevated temperature would result in drier soil conditions. Amount of water supplied was calculated based on the 10-year average monthly precipitation recorded in the nearby station of Deurne, Antwerp, Belgium. Communities were watered three times per week individually by means of a drip irrigation system. Profile probe tubes that fitted a PR2 soil moisture sensor (Delta-T Devices Ltd., UK) were installed in each community and soil water content (SWC) was monitored every 10 days during the experiment. CO_2 fluxes (net ecosystem exchange of CO_2, total ecosystem respiration, and soil respiration) were measured in three periods lasting approximately five consecutive days and starting from July 16, August 13, and September 17.

CO_2 fluxes were measured on the same four randomly selected communities per soil treatment and climate scenario located in different chambers and in three additional communities different in each period, which were afterwards harvested to evaluate above-ground and below-ground biomass. Since photosynthetic photon flux density (PPFD) is the main factor driving photosynthesis, net ecosystems CO_2 exchange (NEE) were measured at varying PPFD from 9:00 until 18:00. Measurements were collected using a closed gas exchange system with a transparent polycarbonate cuvette that covered and sealed the entire container surface (cylinder of 25 cm diameter and 60 cm height). The cuvette was coupled with an EGM-4 infrared gas analyzer (PP Systems, Hitchin, UK). NEE was recorded followed by a measurement of total ecosystem respiration (TER) by darkening the cuvette with a black cloth, preventing in this way photosynthesis. Gross photosynthesis (GPP) was estimated as GPP = NEE – TER. Below-ground respiration (R_{soil}) was measured in small PVC chambers (8 × 5 × 1.5 cm) installed on a strip of bare soil. R_{soil} was then used to calculate above-ground respiration (R_{above}) as R_{above} = TER – R_{soil}. GPP – PPDF curves were obtained for the different measurement periods by fitting a rectangular hyperbola through the data: $GPP = (QE \times GPP_{max} \times PPFD) / (QE \times PPFD + GPP_{max})$, where QE is the quantum efficiency and GPP_{max} is the maximum gross primary productivity. Above-ground and below-ground biomass was collected immediately after CO_2 fluxes measurements and additionally at the beginning of November (end of the experiment). Biomass was then subdivided by species and expressed in terms of total g per unit surface area (m^2) and also in terms of g per individual. Total below-ground biomass was estimated from 12 soil cores per container collected with a 2.0 cm diameter soil probe. The entire soil profile was sampled and divided into four depths: 0-9, 10-18, 19-27 and 28-36 cm. The frequency and intensity of AM fungal colonization was estimated according to method describe in Plenchette and Morel (1996). Other measurements at the plant scale collected at the harvest time included species survival, total leaf area and specific leaf area. Analyses of leaf nitrogen (N) carbon (C) concentration (both expressed as % dry mass) and of phosphorous (P) concentration were carried out for plants harvested in November.

In 2008 a new mesocosm study was established (**study 3**). The objective was to determine the influence of an AMF community on carbon and nitrogen allocation, productivity and community structure of grassland communities, both under the current climate and a future climate scenario. Similar measurements as the one described in study 1 for communities' CO_2 flux measurements, above- and below-ground biomass, and leaf N and P relations were carried out also in 2008. Additionally, ecosystems were labeled with ^{13}C and ^{15}N during three times in the growing season. This would allow obtaining detailed information on C and N allocation to AMF and different plant compartments. To better study the AMF effects on CO_2 fluxes containers were divided in two compartments: (a) plant compartments, with an assemblage of the same plant species used in study 1 and 2 but using only two instead of three individuals per species and (b) a bare soil compartment The two parts were separated below-ground by a 25 µm mesh, allowing AMF hyphae to grow also in the

bare soil but excluding roots. Half of the containers were inoculated with AMF, the other half with sterile inoculum to keep them AMF free. In total four treatments were compared (a) plants in soil, containing AMF, (b) plants in AMF free soil, (c) bare soil containing AMF, (d) bare AMF free soil. All four treatments were compared in the two climate scenarios. For the mycorrhizal inoculum two AMF species were used: *Glomus fasciculatum* MUCL 46100 and *Glomus intraradices* MUCL 41833, kindly provided by GINCO (± 40 root fragments per g of inoculum, 85% frequency of root colonization, Plenchette and Morel, 1996). Inoculum was added to the soil in which the plants were sown before transplantation to the containers. Inoculum, consisted of mycorrhized or sterile lava and mycorrhized or sterile root fragments. The amount of inoculum was approximately 0.5 g per plant. During the transplant every plant received again 1 g of mycorrhizal or sterile inoculum.
To be able to measure CO_2 fluxes on half of the containers (vegetation part) a special cuvette was designed with similar feature as the one used during previous studies but able to seal only half of the container. Fluxes were measured during three measurement periods in July, August and September, each lasting approximately 6 days. After every CO_2 flux measurement period, the plant compartment of the ecosystems was labeled with ^{13}C and ^{15}N. ^{13}C labeling was achieved by injecting 99% $^{13}CO_2$ into a closed cuvette, placed on the vegetation until 1200 ppm was of CO_2 were reached. After the pulse, the cuvette remained on the ecosystem until CO_2 concentration reached the ambient level again (approximately 380 ppm).

Soon after the CO_2 pulsing, ^{15}N was added in the plant compartment to the soil surface as a solution with 99 % ^{15}N $^{15}NH_4{}^{15}NO_3$ (50 kg ha^{-1} of N). One week after the labeling above-ground biomass per species was harvested. To estimate total below-ground biomass and AMF spore count, 18 soil cores were taken at two different depths (0-18 and 18-36 cm) in the planted compartments to have a sufficient amount of roots. In the bare soil compartments 7 soil cores were taken at two different depths.

^{13}C and ^{15}N determination on plant leaves, stubbles, roots and soil are currently carried out at UC Davis Stable Isotope Facility, California, USA. To obtain quantitative information on ^{13}C allocation to AMF, signature fatty acids will be analyzed in cooperation with Prof. Pål Axel Olsson from the Department of Ecology, Lund University, Sweden.
Besides frequency and intensity of AMF root colonization, hyphal length in soil samples will be estimated in cooperation with Prof. Matthias Rillig, Plant ecology group, Freie Universität Berlin, Germany.

Microcosm experiments

The mycelium donor plant (MDP) *in vitro* culture system proposed here allowed for fast, extensive and homogenous colonization of plant roots at the seedling stage It was demonstrated that an AM fungus symbiotically attached to a donor plant is a powerful source of inoculum, allowing for the fast and heavy *in vitro* mycorrhization of seedlings. The plantlets were colonized within 3 days of growth in the extraradical mycelium network. Within 6 days of contact, they were able to reproduce the fungal life cycle after transfer onto fresh medium, with the production of a dense extraradical mycelium bearing a high number of spores.

Details of the new methodology are given in annexe 2.1:

- Voets et al. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. Submitted to Mycorrhiza.

Experiment 1: Effects of eCO_2 and e°T on AMF development

- Short-term time dynamics study of root colonization of *M. truncatula* plantlets grown in an extraradical mycelium network

Plant and fungal growth variables are presented in Tables 5 and 6, respectively. During the whole course of the experiment, the plants grew significantly as shown by all plant parameters measured, except for the shoot length. No differences were observed between plants exposed to either ambient or eCO_2 and e°T conditions at the different time of observations (i.e. 4, 8, 12 and 16 days after the introduction of the plantlets into the mycelium network), except at day 16 where shoot dry weight of plants exposed to elevated CO_2 and temperature was significantly higher as compared to the plants in the ambient conditions. The number of spores and hyphal length produced under ambient conditions or eCO_2 and e°T conditions did not differ at any observation time, except at day 8 where the number of spores in the Petri plates exposed to elevated CO_2 and temperature was significantly higher as compared to the ones in the ambient conditions. Concerning root colonization, no difference between the treatments was observed for any parameter measured and at any day of observation.

The number of leaves was not significantly different at week 0, 1 and 2, but starting from week 3 to week 8, the number of leaves of the plants exposed to ambient conditions was significantly higher than that of the plant grown in eCO_2 and e°T conditions. Shoot and root length did not differ between the two groups of plants at any date. Concerning the production of spores, at week 1, a higher number of spores were counted when plants were exposed to eCO_2 and e°T conditions. However, this initial tendency was not observed thereafter as any significant differences were observed at week 2 to 8. The hyphal length was observed to be significantly higher at week 2, 6 and 8 when plants were exposed to eCO_2 and e°T conditions. At week 8, the hyphal length in these conditions was 17678 ± 1610 cm, while under ambient conditions; the hyphal length reached only 11115 ± 1575 cm. (For details see annex 1.10)

Table 5. Growth variables of *Medicago truncatula* plantlets, grown in an extraradical mycelium network under either ambient or elevated CO2 (eCO2) and temperature (e°T) conditions for 4, 8, 12 and 16 days, estimated by the number of leaves, shoot and root length (cm), and shoot and root fresh (FW) and dry (DW) weight, 4, 8, 12 and 16 days of exposition. The control treatment corresponds to the *M. truncatula* plantlets harvested prior to introduction into an extraradical mycelium network. Six replicates were considered per treatment.

Days after	Shoot length (cm)		Number of leaves		Root length (cm)		Shoot FW (mg)		Shoot DW (mg)		Root FW (mg)		Root DW (mg)	
introduction	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T
4	2,5 ± 0,2 a	2,5 ± 0,4 a	9,5 ± 0,9 a	10,5 ± 0,7 a	50 ± 5,9 a	63 ± 5,6 a	98 ± 9,5 a	128 ± 14 a	20 ± 1,8 a	26 ± 3,5 a	140 ± 13 a	180 ± 17 a	6,2 ± 0,8 a	7,6 ± 0,7 a
8	2,8 ± 0,2 a	3,1 ± 0,4 a	12 ± 0,0 a	13 ± 0,6 a	112 ± 10 a	114 ± 17 a	155 ± 8,3 a	190 ± 15 a	28 ± 1,7 a	38 ± 4,3 a	306 ± 26 a	302 ± 38 a	12 ± 1,1 a	13 ± 1,8 a
12	2,4 ± 0,2 a	2,6 ± 0,1 a	21 ± 1,7 a	21 ± 0,9 a	222 ± 15a	182 ± 22 a	257 ± 10 a	289 ± 20 a	55 ± 3,7 a	61 ± 7 a	652 ± 53 a	571 ± 46 a	39 ± 3,4 a	35 ± 3,3 a
16	2,1 ± 0,1 a	2,0 ± 0,1 a	26 ± 0,6a	22 ± 2,6 a	159 ± 12 a	150 ± 16 a	295 ± 17 a	326 ± 22 a	73 ± 3,4 a	86 ± 4,1 b	896 ± 48a	874 ± 33 a	56 ± 3,6 a	59 ± 0,9a

a Values (means ± SE) within the same column followed by an identical letter are not significantly different ($P \leq 0.05$).

Table 6. *Glomus intraradices* growth variables estimated by the number of spores, hyphal length, and frequency (%F), intensity (%) arbuscular (%A), vesicular (%V) and hyphal (%H) colonization of roots of *Medicago truncatula* plantlets, grown in an extraradical mycelium network under either ambient or elevated CO2 (eCO2) and temperature (e°T) conditions for 4, 8, 12 and 16 days. Six replicates were considered per treatments.

Days after	Number of spores		Hyphal lenght		%A		%V		%H	
introduction	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T	Ambient	eCO2 & e°T
4	0,2 ± 0,2[a] a	21 ± 11 a	41 ± 11 a	53 ± 14 a	1,8 ± 0,7 a	1,1 ± 0,4 a	9,4 ± 1,9 a	6,4 ± 1,4 a	31,2 ± 3,8 a	25,4 ± 4,0 a
8	88 ± 40 a	444 ± 142 b	232 ± 90 a	430 ± 178 a	2,9 ± 1,2 a	8,4 ± 3,2 a	11,6 ± 2,9 a	9,8 ± 3,0 a	28,1 ± 6,0 a	29,4 ± 6,5 a
12	489 ± 256 a	1030 ± 216 a	657 ± 307 a	1213 ± 319 a	6,7 ± 1,8 a	13,2 ± 3,7 a	7,4 ± 2,1 a	14,3 ± 3,6 a	28,0 ± 8,2 a	46,3 ± 9,6 a
16	1273 ± 292 a	1820 ± 555 a	1454 ± 319 a	2374 ± 630 a	13,1 ± 2,5 a	14,8 ± 4,4 a	12,9 ± 5,4 a	19,9 ± 3,9 a	38,3 ± 9,2a	58,0 ± 12,1 a

a Values (means ± SE) within the same column followed by an identical letter are not significantly different ($P \leq 0.05$).

Long-term dynamics study of spore production and extraradical mycelium development from *M. truncatula* plantlets grown in a mycelium network

The table 7 presents the results of the plant growth parameters of *Medicago truncatula* plantlets colonized for 12 days into a mycelium network and then grown for 8 weeks in mono-compartmental HAM-P *in vitro* culture systems in either ambient or elevated CO_2 (eCO_2) and temperature (e°T). No significant differences were observed for any parameters between these two groups. In table 7, the fresh and dry weight of the fungal biomass is also indicated. No differences were observed between the ambient and eCO_2 & e°T conditions for these parameters.

Table 7. Root, shoot and hyphae fresh (FW) and dry weight (DW) eight weeks after mycorrhizal *M. truncatula* plantlets were transferred to half-closed arbuscular mycorrhizal – plant (HAM-P) *in vitro* cultures systems and placed either ambient or elevated CO_2 (eCO_2) and temperature (e°T).

	Shoot FW	Shoot DW	Root FW	Root DW	Hyphae FW	Hyphae DW
Ambient N[a]=10	713 ± 28 a[b]	199 ± 9 a	2529 ± 116 a	240 ± 7 a	21 ± 5 a	2,01 ± 0.39 a
eCO_2 & e°T N=9	702 ± 40 a	202 ± 13 a	2879 ± 200 a	242 ± 11 a	37 ± 7 a	3,39 ± 0.67 a

[a] Number of replicates
[b] Values (means ± SE) within the same column followed by an identical letter are not significantly different ($P \leq 0.05$)

In table 8, the densities of anastomoses within and between hyphae are presented. Anastomoses within a same hyphae was significantly lower under eCO_2 and e°T conditions, while no differences were observed for anastomoses formed between hyphae.

Table 8. Number of anastomones per centimetre of hyphae within and between hyphae is presented eight weeks after mycorrhizal *M. truncatula* plantlets were transferred to half-closed arbuscular mycorrhizal – plant (HAM-P) *in vitro* cultures systems and placed either ambient or elevated CO_2 (eCO_2) and temperature (e°T).

	Number of anastomoses (anastomoses . cm hyphae^{-1})	
	Within the same hyphae	Between hyphae
Ambient N[a]=10	$4{,}00.10^{-2} \pm 4.39.10^{-3}$ a	$1{,}98.10^{-1} \pm 2.84.10^{-2}$ a
eCO_2 & e°T N=9	$2{,}35.10^{-2} \pm 2.76.10^{-3}$ b	$1{,}89.10^{-1} \pm 1.89.10^{-2}$ a

[a] Number of replicates
[b] Values (means ± SE) within the same column followed by an identical letter are not significantly different ($P \leq 0.05$)

Experiment 2: Effects of eCO_2 and e°T on the transport of N and P by AMF
Results will be available beyond the time frame of the project.

Mesocosm experiments

Results of study 1: AMF root colonization was mainly concentrated in roots form the 0-9 cm soil layer where most of the root biomass was located, while it decreased considerably with soil depth; therefore only results relative to the 0-9 cm soil layer are discussed. Frequency of AMF colonization in roots calculated as the percentage of root segments that contained either hyphae, arbuscules or vesicles was significantly higher in *T+CO₂* than in *Amb* (ANOVA, P = 0.002 for climate scenario) mainly from August until November (Table 9). Although not very pronounced, frequency of

colonization in communities in *Amb* was overall successful particularly considering the young ecosystems used in this study, reaching maximum values of 10% and 27.5% in *Amb* and *T+CO₂* communities, respectively, at the end of the season (Table 9). Similarly, intensity of AMF root colonization (the abundance of vesicles, hyphae and arbuscules in each root segment) was positively affected by the future climate scenario and AMF were more abundant in root fragments of *T+CO₂* than *Amb* communities (ANOVA, P = 0.045 for climate scenario) and had the tendency to increase during the season from August till November in *T+CO₂* (marginally significant measurement time, P = 0.062, Table 9).

Table 9. Average AMF root colonization (frequency and intensity) of grassland communities exposed to ambient air temperature and 375 ppm of CO_2 concentration (*Amb*) or to ambient air temperature + 3 ºC and 620 ppm of CO_2 (future scenario, *T+CO₂*) in roots sampled in the 0-9 cm layer of soil during different periods of the growing season.

Harvest period	Frequency (SE) %		Intensity (SE) %	
	Amb	T+CO_2	Amb	T+CO_2
July	7.8 (4.8)	6.7 (6.7)	10.0 (5.8)	10.0 (10.0)
August	3.3 (3.3)	22.2 (7.8)	3.3 (3.3)	19.6 (8.0)
September	3.3 (1.9)	34.4 (10.6)	6.7 (3.3)	23.0 (7.2)
November	10.0 (3.6)	27.5 (6.3)	20.0 (5.8)	30.7 (6.0)

Community above-ground productivity significantly increased over the growing season from July to November (P < 0.0001) similarly in communities growing in the two climate scenarios (annex 1.11a, P = 0.427) and was not affected by the presence of AMF in any of the harvest periods (annex 1.11a, P = 0.332). As for total ecosystem, above-ground biomass of individual species also increased significantly from July to November (P < 0.0001) and was not affected by the climate scenario (P = 0.580, annex 1.12). Species clearly differed in their biomass (P < 0.0001) and a consistent dominance of *L. perenne* and *P. lanceolata* was observed throughout the growing season (annex 1.12). Species were also positively affected by the presence of AMF (P < 0.0001, annex 1.12, small insert) regardless of the climate. These differences were significant for *M. lupulina* that had a higher biomass in the presence of AMF starting from August and for *P. pratensis* although only to a small extent and limited to the period of September (post-hoc analysis of the significant species × soil treatment ×time interaction P = 0.0275).

Root biomass was highest in the first layer of soil with approximately 44% of the total root biomass located in the 0-9 cm layer and gradually decreased with soil depth (P < 0.0001 for soil depth, annex 1.13) and the same pattern was observed in all the periods considered. Generally the climate scenario did not affect root biomass production (P = 0.092) with the exception of the last harvest where *NM* root biomass was 38% higher in *T+CO₂* than *Amb* (post-hoc test of the significant scenario × soil treatment ×time interaction, P = 0.0226, annexes 1.11b and 1.13 d). Also by the end of the season, the presence of AMF negatively affected root biomass but only under *T+CO₂* scenario resulting in 60% lower biomass in *AM* communities compared to *NM* ones (first 3 layers of soil, annex 1.13). As a result of the effect of AMF on below-ground biomass, total community biomass (combined above- and below-ground) was also negatively affected by AMF during the last harvest in the *T+CO₂* climate and was significantly higher in *T+CO₂* than *Amb* in the *NM* communities (significant climate × soil treatment × time interaction, *P* = 0.041, annex 1.11c).

In order to evaluate differences in GPP due to the climate scenarios and the presence of AMF, data were log transformed to achieve linear distribution. An ANCOVA analysis with PPFD as covariate and climate, soil treatment and time as fixed factors was performed. GPP was mainly influenced by PAR and only marginally by the climate scenario which determined slightly higher GPP under *T+CO₂* for both *NM* and *AM* treatments (annex 1.14), ANCOVA with PPFD as a covariate, P < 0.0001 and P = 0.0801 for PPFD and scenario, respectively). GPP was not significantly affected by the presence of

AMF and was not significantly different between measurement periods (ANCOVA, P = 0.223 and P = 0.127 for soil treatment and measurement period, respectively), decreasing from July to September (different intercepts for the measurement period, P = 0.0408). Regressions for R_{above} and R_{soil} were also fitted separately for the two climate scenarios and soil treatments in each of the measurement periods (12 regressions in total). R_{above} and R_{soil} calculated at the growth temperature of 20 ºC and 23 ºC for the *Amb* and the *T+CO₂* scenario, respectively, were not affected by the climate scenario, the soil treatment, or different between measurement periods but varied only as a function of T_{air} or T_{soil} (ANCOVA with T_{air} or T_{soil} as covariate, P < 0.0001 and P = 0.0004 for T_{air} and $T_{soil,}$ respectively). Although similar slopes were detected for the climate scenarios, there was a tendency to a lower R_{above} at a given air temperature under *T+CO₂* than in *Amb* (marginally different intercepts for climate scenario, P = 0.065). In general the contribution of R_{soil} to the total ecosystem respiration was approximately 15%.

The N pool at the species level was calculated multiplying the N concentration of individual species by the species above-ground biomass. Similarly to the significant trends observed in species above-ground biomass, also the N amount per species was not affected by the climate scenario (P = 0.236) but was different depending on the species (P < 0.0001) and increased in the presence of AMF (P = 0.003). The differences due to AMF were limited to *M. lupulina* (significant soil treatment × species interaction), which contributed to approximately 9 and 14% to the community N pool in *Amb* and in *T+CO₂*, respectively while in *NM* treatment its contribution was close to zero. Nevertheless, the N amount at the community level was not different between climate scenarios and soil treatments (P = 0.773 and P = 0.593 for climate scenario and soil treatment, respectively) with averages (±SE) of 17.8(±1.4) and 19.9 (±1.1) g N m^{-2} in *NM Amb* and *T+CO₂* and averages of 20.4 (±3.3) and 19.5 (±1.2) g N m^{-2} for *AM* in *Amb* and *T+CO₂*.

Results of study 2: the frequency of AMF root colonization was not significantly affected by the climate scenario (P = 0.276), except during the last sampling period, when it was significantly lower in *T+CO₂* than in *Amb* (Table 10, *a posteriori* analysis of the significant climate scenario × date interaction, P = 0.011). However, because root length was slightly higher in *T+CO₂* than in *Amb* (9.18 and 14.21 m cm^{-3} in *Amb* and *T+CO₂*, respectively, in the first 9-cm layer), this lower root colonization rate in *T+CO₂* was not reflected in the total length of colonized roots (3.08 and 2.51 m cm^{-1} in *Amb* and *T+CO₂*, respectively). The frequency of AMF root colonization decreased with soil depth (P = 0.001) and was on average 35% lower in the deepest soil layer (28-36 cm) than in the first two layers (0-9 and 10-18 cm). Intensity of root colonization, on the other hand, was enhanced under *T+CO₂* (approximately 28%, P = 0.043, Table 10). However, differences between climate scenarios were only significant in some of the soil layers in some of the periods. For example, higher intensity of root colonization in September in *T+CO₂* was mainly due to the higher colonization observed in the 19-27 cm soil layer (*a posteriori* analysis of the significant climate scenario × soil depth × date interaction, P = 0.014, data not shown).

Table 10. Average AMF colonization (frequency and intensity) of roots from four soil layers (0-9, 10-18, 19-27 and 28-36 cm) sampled in grassland communities during the growing season. Grassland communities were exposed to ambient air temperatures and 375 ppm of CO_2 (*Amb*) or to 3 ºC above fluctuating ambient air temperatures and 620 ppm of CO_2 (future scenario, *T+CO₂*). Means (SE) of three replicates in July, August and September and of four replicates in November.

Harvest period (day of the year)	Frequency (SE) %		Intensity (SE) %	
	Amb	*T+CO₂*	*Amb*	*T+CO₂*
July (201)	13.6 (1.5)	13.6 (2.2)	13.1 (0.9)	12.6 (1.4)
August (230)	11.1 (3.4)	13.1 (1.7)	8.3 (1.6)	13.9 (2.3)
September (267)	11.4 (1.6)	10.1 (2.1)	9.2 (0.8)	19.7 (7.2)
November (310)	25.0 (2.7)	14.7 (2.3)	10.9 (0.5)	11.8 (1.7)

Considering all harvest periods, average total biomass was 25% higher in *T+CO₂* than in *Amb*. The difference was significant from August on.(P = 0.0104; annex 1.15a) and became the largest (41%) in the last sampling period. It was primarily due to above-ground biomass, which was positively affected by climate (P = 0.007) from August on (Annex 1.15b), whereas total below-ground biomass was not (P = 0.250, annex 1.15c). Also for the individual species, above-ground biomass was significantly higher in *T+CO₂* (P = 0.0074, annex 1.16). Among the species *L. perenne* and *P. lanceolata* showed the highest biomass values and the *a posteriori* analysis of the significant species × date interaction (P = 0.0009) indicated that both *M. lupulina* and *L. corniculatus* performed better later in the season (Annex 1.16).
Root biomass per soil layer (mg root cm^{-3} of soil, annex 1.17) increased significantly over the season (P = 0.0025) and was higher in the 0-9 cm soil layer compared to all the other layers (P < 0.0001). Although root biomass was not significantly affected by climate scenario, root length at the end of the season tended to be higher in *T+CO₂* than in *Amb* and this effect was borderline significant in the upper soil layer (P = 0.07). Moreover, root biomass of the 0-9 cm soil layer at this time was nearly two-fold higher in *T+CO₂* than in *Amb* (Annex 1.17d), while root diameter and specific root length were similar in both treatments.

As expected, PPFD explained most of the variation observed in GPP (ANCOVA, P < 0.0001), which was also reflected in the goodness of fit of the individual regressions (Annex 1.18). GPP was positively influenced by climate (ANCOVA, similar slopes but higher intercepts, P = 0.011, annex 1.18) even though in September GPP approached similar maximum values in both climate scenarios (Annex 1.18c). Overall, GPP increased from July to September (different intercepts for the measurement periods, P = 0.048).

Similarly to GPP, also R_{above} increased during the season (similar slopes between measurement periods but greater intercepts, P = 0.045). Differences between climate scenarios, on the other hand, were not significant (ANCOVA, P = 0.622). Most of the variation in R_{above} was explained by the covariate air temperature (P = 0.004). Although differences between climate scenarios were not significant, R_{above} was about 10% higher in *T+CO₂* than in *Amb* during July and August and even two times higher during September. Soil respiration for the three measurement periods combined increased with soil temperature (ANCOVA, P < 0.0001), but did not differ between the two climate scenarios (P = 0.689). On average, R_{soil} was 0.71±0.05 and 0.88±0.16 µmol CO_2 m^{-2} s^{-1} in *Amb* and *T+CO₂*, respectively. On the other hand, the intensive measurement campaign for R_{soil} at the end of the season revealed a tendency toward higher BR at growth conditions in *T+CO₂* than in *Amb* (P = 0.066; annex 1.19), and even the BR at 10 °C for both climate scenarios tended to be higher in *T+CO₂*. Last, the temperature sensitivity of R_{soil} at the end of the season was very similar for both climate scenarios, with a weighted average Q_{10} of approximately 2.8 (Annex 1.19).

In November, leaf C and N concentrations were lower in *T+CO₂* than in *Amb* and varied across the species (Table 11). The C:N ratio was significantly higher in *T+CO₂* than in *Amb* in all the species considered, except in *L. corniculatus*, which exhibited the smallest changes in C and N concentrations (Table 11). In spite of the lower N concentration found in *T+CO₂*, total acquired N per species did not differ between the climate scenarios (Table 11). Root N concentration was not significantly different between the treatments, whereas root C concentration tended to be higher in *T+CO₂* than in *Amb*. Nonetheless, the C:N ratio was not significantly affected by climate scenario. Total ecosystem N content (considering both above- and below-ground components) was significantly higher in *T+CO₂* than in *Amb* (P = 0.022, 13.6±0.6 SE and 11.2±0.5 SE g N m^{-2}).
Leaf phosphorus concentration did not significantly differ between the two climate scenarios and, given the higher biomass observed in *T+CO₂* than in *Amb*, the total P content per species was thus higher in *T+CO₂* than in *Amb*. This effect was most pronounced in the leguminous species (Table 11). Likewise, the total amount of above-ground P was significantly higher in *T+CO₂* than in *Amb* (P = 0.023), reaching values of 2.8±0.1 and 2.2±0.1 g P m^{-2} in *T+CO₂* and *Amb*, respectively.

Table 11. Above-ground nitrogen (N), carbon (C), and phosphorus (P) concentration, C:N ratio, and total N and P content in species harvested in November (day of the year 310). Means (SE) of four replicates. Species: *Lolium perenne* (*Lp*), *Plantago lanceolata* (*Pl*), *Rumex acetosa* (*Ra*), *Lotus corniculatus* (*Lc*), *Poa pratensis* (*Pp*), *Medicago lupulina* (*Ml*).

		Species						Significance (P-value)	
		Ml	*Lc*	*Ra*	*Pl*	*Lp*	*Pp*	**Climate scenario**	**Species**
C concentration (%)	*Amb*	44.2 (0.4)	44.5 (0.6)	41.0 (0.6)	41.2 (0.3)	40.3 (0.2)	40.3 (0.6)	**0.045**	**< 0.0001**
	T+CO₂	41.7 (1.8)	43.5 (0.4)	39.4 (0.7)	39.4 (1.1)	40.2 (0.2)	42.1 (0.3)		
N concentration (%)	*Amb*	3.2 (0.2)	2.6 (0.1)	2.4 (0.3)	1.6 (0.1)	1.3 (0.1)	1.3 (0.1)	**< 0.0001**	**< 0.0001**
	T+CO₂	2.4 (0.4)	2.4 (0.1)	1.4 (0.2)	1.1 (0.1)	1.1 (0.0)	1.2 (0.1)		
C:N ratio	*Amb*	13.9 (0.7)	17.5 (0.4)	17.8 (2.3)	26.8 (1.8)	30.6 (1.5)	32.3 (3.1)	**< 0.0001**	**< 0.0001**
	T+CO₂	19.3 (3.9)	17.8 (0.5)	29.7 (3.6)	36.7 (2.1)	37.3 (1.4)	36.9 (3.0)		
N content (g N m^{-2})	*Amb*	1.2 (0.3)	0.8 (0.1)	0.9 (0.4)	3.2 (0.3)	2.5 (0.2)	0.3 (0.1)	0.612	**< 0.0001**
	T+CO₂	2.1 (1.1)	1.4 (0.7)	0.4 (0.1)	2.8 (0.6)	2.7 (0.5)	0.3 (0.0)		
P concentration (mg P g DW^{-1})	*Amb*	4.7 (0.7)	5.0 (0.7)	4.6 (0.4)	4.2 (0.2)	4.2 (0.1)	3.3 (0.6)	0.565	**0.0002**
	T+CO₂	5.0 (0.7)	6.4 (0.6)	4.7 (0.4)	3.7 (0.6)	4.1 (0.1)	3.0 (0.3)		
P content (g P m^{-2})	*Amb*	0.2 (0.0)	0.1 (0.0)	0.2 (0.1)	0.9 (0.1)	0.8 (0.1)	0.1 (0.0)	**0.046**	**< 0.0001**
	T+CO₂	0.5 (0.2)	0.3 (0.1)	0.2 (0.0)	0.9 (0.1)	1.0 (0.1)	0.1 (0.0)		

Results of study 3: for this study only the data on total above-ground ecosystem biomass are shown. Other data (e.g., ecosystems CO2 fluxes, ^{15}N and ^{13}C allocation etc…) are currently processed.
Analysis of variance (ANOVA) of ecosystem biomass was performed in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) using the mixed procedure described in Littell *et al.* (2006). The ANOVA included the climate scenario (*Amb* and *T+CO₂*), the soil treatment (*AM* and *NM*) and the time period as fixed factors and their interactions. Biomass data were log transformed to achieve normality. Overall above-ground biomass was positively influenced by the future climate scenario ($F_{1, 67} = 10.19$, $P = 0.002$). Mycorrhizae also enhanced ecosystems biomass both under *Amb* and *T+CO₂* determining a significantly higher biomass in *AM* compared to *NM* during the fall, in the October and November harvests (*a posteriori* analysis of the significant soil treatment × date interaction, $F_{3, 67} = 3.24$, $P = 0.027$) (Annex 1.20).

WP 5 - Modeling of the processes describing C balance in grassland ecosystems

The ANAFORE model (ANAlysis of FORest Ecosystems) is a fully mechanistic stand-scale model, consisting of a combination of sub-models. ANAFORE includes the effects of additional factors affecting growth such as elevated atmospheric CO_2, fertilization, drought, ozone, and temperature extremes. Stomatal conductance, photosynthesis and transpiration are simulated on half-hourly or 10 minute basis for up to 100 leaf layers of each plant species. Allocation of the cumulated C gain is calculated on a daily basis, using the improved pipe theory (Deckmyn et al., 2007, Verbeeck et al.,

2007). The soil sub-model in the ANAFORE has been adapted from the Century model (Parton et al., 1987) and Thornley's grassland model (Thornley, 1998) and includes active roles of the bacteria, mycorrhizae and other fungi. As in the case of the other applications of the ANAFORE model (e.g. single tree species in forest stands), was adapted to simulate individual species in the grassland communities. Modeling of photosynthesis, transpiration and growth of annual species has been completed over the course of the last year. An option has been included in the model to either simulate the grassland communities as a canopy (including percentage of ground cover) or in terms of number of individual plants per m^2. The ANAFORE model has now been improved so that it simulates competition between plants in terms of light, water and nutrients. In total the model uses 128 parameters per species. Since many of these parameters are not always available and since we want to have an idea of the uncertainty level, a Bayesian optimalisation technique has now been included in the model. Once you have a starting dataset available (including a range for each of the 128 parameters, eventually from the literature), any output data and their uncertainties can be used to find an optimal fit, and calculate an improved input dataset (narrower ranges). This improved input dataset will then generate output including an uncertainty interval. This means that a stronger fit to the better data is obtained. One optimalisation run of the model takes about one week. It is the plan for the next phase to do this optimalisation run, once we have an ideal and more or less complete dataset available.

5. CONCLUSIONS AND RECOMMENDATIONS

WP 1 - Assessment of the AMF biodiversity in selected Belgian grasslands

Preliminary observations of the soil collected at the five different sites showed that spores of AMF were present. However, until further characterisation (taxonomy and phylogeny), assessment of the biodiversity of AMF could not be performed. Germplasm collection was initiated. New attempts for *in vitro* culture of AMF will be conducted starting from spores coming from the monospecific-AMF pot cultures. Cultures are running and because of the slow development of AMF, data are expected to be fully collected by the end of year 2.

WP 2 - Role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands and their feedbacks on AMF

The results presented in this WP highlighted the complexity of carbon labeling studies and the different pathways involved in the transfer of carbon from one autotrophic plant to another, via a common AM fungal network. No direct carbon flow could be observed from a donor to a receiver plant via the AM fungal network under *in vitro* conditions linking two autotrophic plants. In the three different types of C-sinks created, the C that was transferred from the donor plant to the receiver plant via the AM fungal hyphae seemed to remain in the roots of the receiver plant, and was not transferred to the receiver's plant tissues.
These results do not exclude that a C-flow is possible between plants, with other plant-fungus combinations. More experiments are therefore needed, with receiver plants that present a higher sink than the plants used in our experiments. For this, we can think about achlorophyllous plants.

WP 3 - Impact of elevated CO_2, temperature and water availability above- and below-ground biodiversity, AMF-plant associations and C cycle

At the microcosm scale

The mycelium donor plant (MDP) *in vitro* culture system allowed for fast, extensive and homogenous colonization of plant roots at the seedling stage. Thanks to the continuous carbon flow into the mycelium network and to the density of the mycelium, with hundreds of hyphae growing out of the donor plant roots, colonization levels of more than 50% could be reached in seedlings after 3 days of growth in the mycelium. These colonization levels have never been obtained in any *in vitro* study before. This system offers wide research and application possibilities. Various economically important monocots (maize, banana) as well as herbs (clover, *Plantago*) and shrubs (vineyard) have been successfully colonized in their seedling stage using this system with *M. truncatula* as donor plant. This might offer a broad range of research possibilities for which either homogenous highly-colonized seedlings or mass-produced *in vitro* inoculum is necessary.

Effects of eCO_2 and e°T on AMF development

Hyphal length was positively affected by eCO_2 and e°T, and, even though results were not significant ($p=0.0711$ and $p=0.0947$ for fresh and dry weight, respectively), the total biomass of the extraradical mycelium increased by about 70% under eCO_2 and e°T. The number of spores produced under ambient and eCO_2 and e°T were, however, not significantly different. This tends to indicate that eCO_2 and e°T could influence (i) AMF soil exploration and resources foraging, (ii) soil structure by increasing soil aggregation, and (iii) C sequestration by higher chitin production. The fact that spores production was not affected by eCO_2 and e°T, and that hyphal length increased showed that the capacity of AMF to colonize new plants could be higher due to (i) increased the dispersion of the spores and (ii) higher probability of inter-plant mycorrhization.

At the mesocosm scale

In study 1, AMF root colonization was promoted in *T+CO_2* compared to *Amb* (both frequency and intensity were higher). AMF have been previously reported to increase in presence of elevated CO_2 concentration as observed in two Californian annual grasslands (Rillig et al., 1999) or in agricultural crops like sorghum (Rillig et al., 2001) and cotton (Runion et al., 1994). Elevated CO_2 typically promotes photosynthesis and higher allocation of C to the root compartment (Pendall et al., 2004) while on the other side, warming is likely to increase respiration and decomposition rates and to reduce above- and below-ground biomass in grasslands (De Boeck et al., 2008). Although only a tendency toward increasing GPP under *T+CO_2* was observed, allocation to root was promoted in the future climate in absence of AMF, as shown by the higher root biomass observed in the first three soil layers in *NM* in *T+CO_2* than in *Amb* at the end of the season. On the other side, allocation to roots was similar in the two climate scenarios when AMF were present. In spite of similar root biomass, higher AMF colonization rates observed in *T+CO_2* in *AM* communities suggest that C was probably allocated to the AMF pool instead of to the roots. By extending in the soil, the AMF mycelium allows roots to acquire nutrients (i.e. N and P) and its presence may lead to less investment in root biomass.
Species like *M. lupulina* and *P. pratensis* performed better in presence of AMF in both climate scenarios, confirming the important role of AMF in determining plant community composition. The fact that a N-fixer almost completely disappeared from the *NM* treatment in both scenarios did not significantly affected the above-ground N-pool even if *M. lupulina* had one of the highest percentages of N content among the species considered. However, it is expected that on the long-term the loss N-fixers will affect the N cycle as total N input will decrease, stimulating microbial activity and nutrient mobilization. Such mobilization might induce a faster decomposition of soil organic carbon and increase the release of CO_2 in the atmosphere.

In study 2 with the unmanipulated soil, AMF root colonization was not increased by the exposure of grasslands to the future climate. While little or no CO_2 effect was found on AMF development in the case of *Pisum sativum* (Gavito *et al.*, 2000; Gavito *et al.*, 2003) or in 10 herbaceous species with different growth strategies (Staddon *et al.*, 1999), warming generally increased AMF root colonization as observed in an annual grassland (Rillig *et al.*, 2002) and in *P. lanceolata* (Staddon *et al.*, 2004). Such a positive effect of warming on AMF growth was not evident in our experiment, possibly because it was off-set by the lower SWC. The latter was observed by Monz *et al.* (1994) in western wheatgrass exposed to 4 ºC warming. We cannot exclude, however, that some of the AMF taxa in our experiment responded positively to the climate treatment. Exposure to elevated temperature and CO_2 enhanced total biomass production, mainly due to the effect of the future climate on above-ground biomass. Elevated CO_2 was often found to increase grassland production (e.g., Hebeisen *et al.*, 1997; Ainsworth *et al.*, 2003; Nowak *et al.*, 2004) while warming, instead, was reported to significantly reduced plant biomass, in the absence of higher precipitation (De Boeck *et al.*, 2008). In line with the higher above-ground biomass in the future climate scenario, GPP was significantly increased in *T+CO_2*. However, this was not the case during September were GPP_{max} approached similar values in both climate scenario. This could partially be explained by water limitations experienced by the warmed ecosystems, even if overall differences in SWC were small between the climate scenarios. Under simulated warming only, GPP and above-ground biomass in grassland communities were reported to slightly decrease (Saleska *et al.*, 1999; De Boeck *et al.*, 2007). De Boeck *et al.* (2007) observed the largest decrease in GPP during the summer months, when warming-associated drought stress was most prominent compared to other periods. At the end of the season, basal rate of R_{soil} at growth temperature had the tendency to be higher in *T+CO_2* than in *Amb*. Most studies considering elevated CO_2 effects reported an increased R_{soil} (e.g., Zak *et al.*, 2000). Elevated temperatures also have been reported to enhance R_{soil} (Rustad *et al.*, 2001), although in some cases R_{soil} acclimated quickly to the warmer conditions (Luo *et al.*, 2001; Tingey *et al.*, 2006). In our study, there was no complete down-regulation (i.e., homeostasis) of R_{soil} to warming and CO_2 enrichment, as BR under growth conditions increased in *T+CO_2* compared to *Amb*. R_{soil} tended to be higher under the future climate scenario due to the higher root length and biomass in *T+CO_2* in the first soil layer as compared to *Amb*. Our results that newly established grasslands on a soil with a AMF taxa from a Belgian grassland could benefit from the future climate in terms of total growth. However, extra C was allocated mainly to above-

ground plant parts, whereas roots and AMF did not increase under the future climate scenario. Together with the tendency toward increasing R_{soil} in *T+CO₂* as compared to *Amb*, all together the results point towards a negative effect on soil C sequestration when grasslands are exposed to a future climate.

It is currently not clear why in study 2 with the natural AMF community there were climate effects on above-ground biomass while study 1 showed only an effect of climate on below-ground biomass at the end of season although this effect was limited to *NM* communities. One possible explanation could be that in the unmanipulated soil in study 2 nutrients were released at a slower rate compared to the pasteurized treatments in study 1. N was probably not limiting in the pasteurized soil due to the pasteurization treatment itself and species such as *L. perenne* and *P. lanceolata* were able to utilize the available nutrients faster than other species, becoming quickly dominant species in these communities. On the hand, in the unmanipulated soil N-fixers performed better under the future climate conditions, taking full advantage of the elevated CO_2 and temperature.

6. FOLLOW-UP COMMITTEE

During the first phase of the project the members of the follow-up committee contributed with some ideas and recommendation to improve the quality of the different WPs.
In the assessment of the AMF biodiversity in selected Belgian grasslands, the selection of the soils as well as the size of the sampling to assess the AMF biodiversity was made in agreement with the follow-up committee.

In the role of AMF for seedling establishment, plant community structure, diversity and productivity in grasslands and their feedbacks on AMF, the follow-up committee recommended that in the experiments at the mesocom scale the centre core should be sterilized to avoid development of AMF in treatment. All the three treatments should have the same amount of sterilized soil to make sure that the communities have access to similar nutrient contents in the soil. A mesh bag with a large mesh should be included in treatment to allow roots and AM fungi to develop in the centre core. In treatment, where roots of the established community can explore all the soil in the pot, inert material (e.g. small chips used for packaging) should be added to the soil in an amount equal to the same volume of the centre core in order to have communities growing in similar soil amounts. In this way we prevent that established communities are larger than in the other two treatments. The centre cylinder of the pots of treatment should be covered on the top with a mesh to avoid AMF's contamination during winter. It will be of interest to add in the centre core *Rumex acetosa*. In contrast with the other three plant species selected for the centre core (*Lolium perenne*, *Lotus corniculatus* and *Plantago lanceolata*), AMF do not established well with *R. acetosa*. It could be useful to use a non-mycotrophic mutant of *Medicago* (Myc-/Nod+) versus the mycotrophic wild type (Myc+/Nod+). Nod refers to the capacity to form association with N-fixing bacteria. It would be of interest to study the effects of AMFs on seedlings establishment in nutrient-poor vs. nutrient-rich soil.

At the microcosm scale the follow-up committee proposed that we could enrich the donor plant for a longer period by continuous labelling or greater quantities of ^{13}C so more labelled C will be available for the receiver plant. Also they proposed that we could use a non-autotrophic mutant as a receiver plant, which could act as a greater sink for C than an autotrophic plant. Another option is to use a tobacco mutant.

7. PUBLICATIONS / VALORISATION

7.1. Publications

7.1.1. Peer review

- Liesbeth Voets, Isaline Goubau, Pål Axel Olsson, Roel Merckx and Stéphane Declerck. 2008. Absence of carbon transfer between *Medicago truncatula* plants linked by a mycorrhizal network, demonstrated in an experimental microcosm. **FEMS Microbiology and Ecology 65: 350-360.**
- Deckmyn G, Verbeeck H, Op de Beeck M, Vansteenkiste D., Steppe K, Ceulemans R. 2008. ANAFORE: A stand-scale process-based forest model that includes wood tissue development and labile carbon storage in trees. **Ecological Modelling 215: 345–368**
- Liesbeth Voets, Ivan Enrique de la Providencia, Kalyanne Fernandez, Marleen IJdo, Sylvie Cranenbrouck, Stéphane Declerck. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. **Submitted to Mycorrhiza**.
- Costanza Zavalloni, Liesbeth Voets, Hervé Dupré de Boulois, Joke Van den Berge, Sara Vicca, Ivan de la Providencia, Stéphane Declerck, Reinhart Ceulemans, and Ivan Nijs. Arbuscular micorrhizal fungi reverse the productivity response to the future climate of grasslands. **Submitted to Agr. Ecosys. Environ.**
- Sara Vicca, Costanza Zavalloni, Yongshuo Fu, Liesbeth Voets, Hervé Dupré de Boulois, Stéphane Declerck, Reinhart Ceulemans, Ivan Nijs, and Ivan A. Janssens. Arbuscular mycorrhizal fungi can mediate belowground responses to elevated temperatures and CO_2 enrichment. **Submitted to New Phytologist**.
- Costanza Zavalloni, Sara Vicca, Ivan de la Providencia, Hervé Dupré de Boulois, Manu Büscher, Stéphane Declerck, Ivan Nijs, and Reinhart Ceulemans. Warming and CO_2 enrichment promoted above-ground production but not below-ground carbon sequestration in newly established grasslands. **Submitted to Biogeosciences**.

7.1.2. Others

- Voets, 2007. Role of arbuscular mycorrhizal networks on plant interconnection and carbon transfer. PhD thesis at the Université catholique de Louvain, pp: 220.
- Simon A. 2007. Rôle des champignons mycorhiziens à arbuscules dans le transfert de carbone d'une plante autotrophe à une plante myco-hétérotrophe. Mémoire présenté à l'Université catholique de Louvain, pp: 65.
- Goubau, I. 2007. Rôle de champignons mycorhiziens à arbuscules dans le transfert de carbone d'une plante adulte autotrophe à une jeune plantule de la même espèce. Mémoire présenté à l'Université catholique de Louvain.
- Flyer of the Mycarbio project (Annex 1.21)

7.2. Other activities

- Dupré de Boulois, H., Zavalloni, C., Deckmyn, G., Nijs, I., Ceulemans, R., Declerck, S. Mycorrhizae impact on biodiversity and C-balance of grassland ecosystems under changing climate (MYCARBIO). Belgian Biodiversity Platform conference. Conference on Biodiversity and Climate Change. 21-22 May 2007. Brussels, Belgium.

- Dupré de Boulois, H., Declerck, S. *In vitro* mycorrhization of seedlings: prospects for fundamental and applied research. Working Groups 2 and 4 meeting of COST Action 870. 17-19 September 2008. Thessaloniki, Greece.

8. SUPPORT TO THE DECISION

Partners of the project are involved in EU initiatives (COST actions - UCL) and in de GIEC (UA). Through both channels a number of results may be transmitted improving knowledge on the intimate plant/fungi interaction under modified climate conditions and carbon cycling that may help in decision.

Briefly, results of interest (at present) are of two orders

1. We start to have a better insight in the grassland/AMF population relationship under increased temperature and CO_2. This may improve (1) our understanding of below-ground C storage, (2) help decision for ecosystem preservation planning taking into consideration the microbial hidden-half and (3) have a better insight on the ecological significance of above/below-ground interactions under climate change.

2. We started to monitor 5 grasslands (AMF biodiversity) and will check the evolution in the coming years. This will help to assess the impact of climate change on AMF biodiversity evolution.

A third aspect (not –finished yet) is the final refinement and application of the process-based simulation model ANAFORE. The validated model will provide data to plan and evaluate actions and policies in relation to below- and above-ground biodiversity and C cycle in the context of climate change. In particular, this model could be used to estimate C sinks in relation to article 3.4 of the Kyoto Protocol to report and monitor changes in soil C stocks necessary to provide sufficient level of "verifiability" required under this article. This aspect will be considered in detail in the second phase of MYCARBIO.

Finally it is important to note that both teams have submitted in September 2008 a Marie Curie ITN project (AMCLIMATE) joining 10 partners and industrials of EU and Canada to increase our knowledge of the impact of climate change on the above/below-ground interactions. This project coordinated by UCL will (if successful) come in support to MYCARBIO and help decision in many domains from (1) microbial management for e.g., improved plant biodiversity, production and health, resistance to climate stress, ecosysterm preservation, carbon storage, to (2) modelling of the complex interactions between above and below-ground organisms to better comprehend C sinks and storage under climate change.

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10. ANNEXES

Annex 1.1	Schematic representation of the roots and soil sample strategy.
Annex 1.2	*In vitro* culture initiation from spores or colonized roots.
Annex 1.3	Morphological description of *G. mosseae* spores.
Annex 1.4	Morphological description of *Glomus sp* spores.
Annex 1.5	Morphological description of *Glomus sp* spores.
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Annex 1.8	Representation of the AMP *in vitro* experimental system, developed to investigate carbon transfer.
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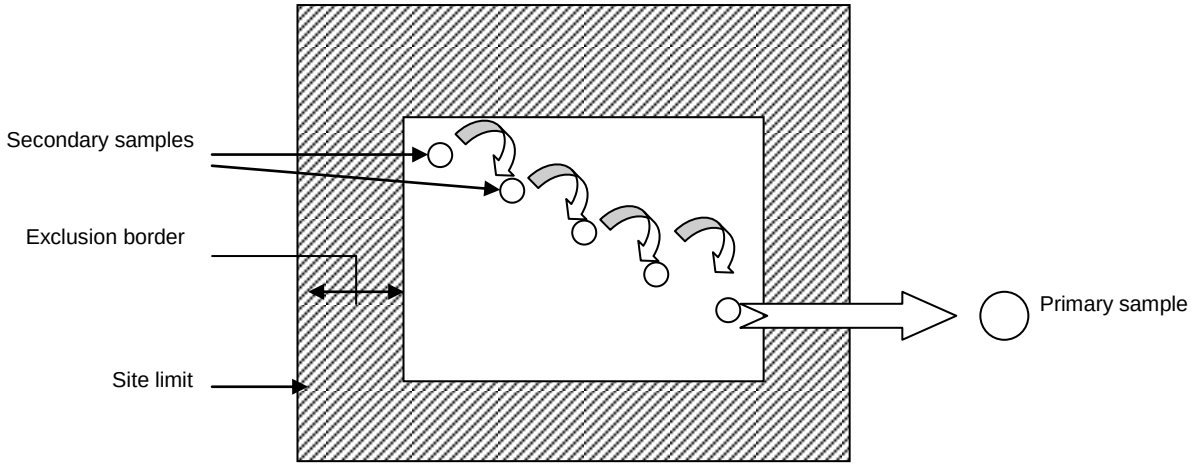
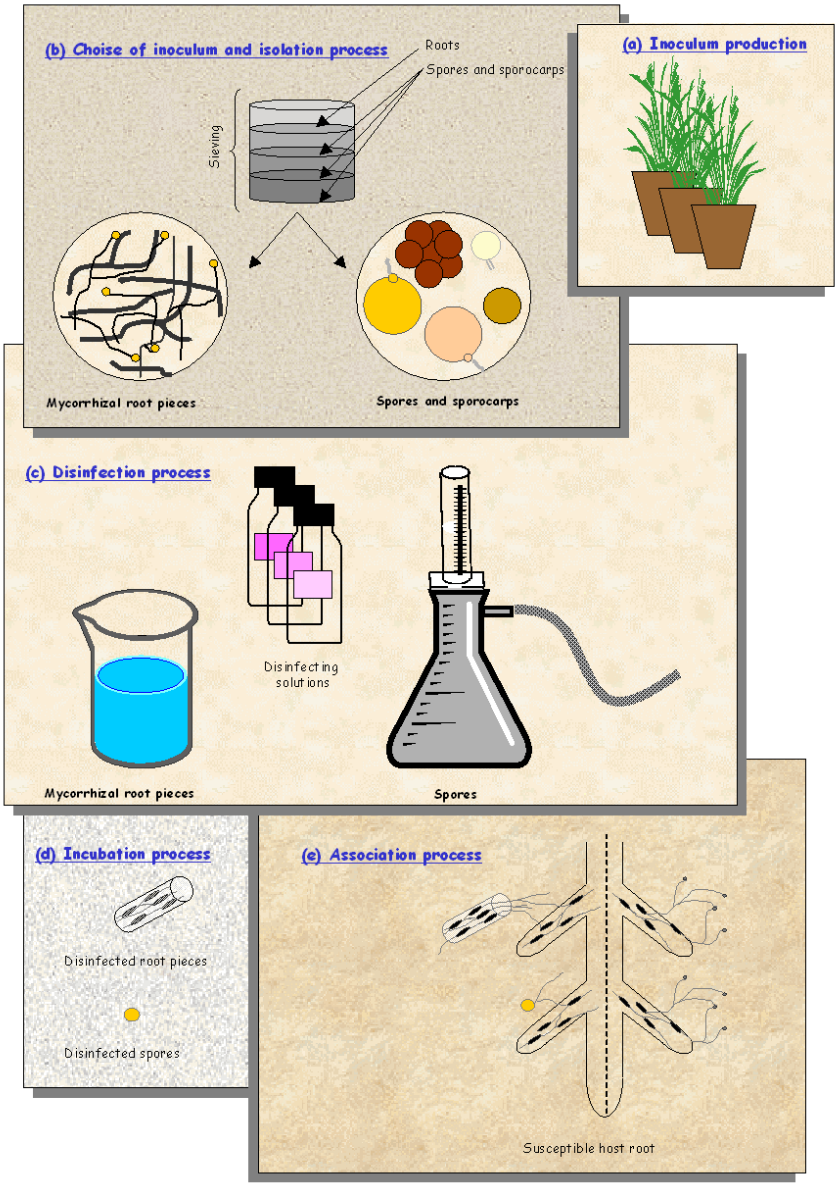
Annex 1.18	Fitted regression curves to the relationship of gross primary productivity (GPP) and photosynthetic photon flux density (PPFD).
Annex 1.19	Weighted mean basal rate of soil respiration and temperature sensitivity (Q_{10}) of grassland communities.
Annex 1.20	Time course of above-ground biomass of grasslands.
Annex 1.21	Flyer of MYCARBIO project.
Annex 2.1	Voets et al. Submitted to Mycorrhiza.
Annex 2.2	Deckmyn, G et al. Ecological modelling. 345-368.
Annex 2.3	Voets et al. 2008. FEMS Microbiology and Ecology. 65 : 350-360.

ANNEXES

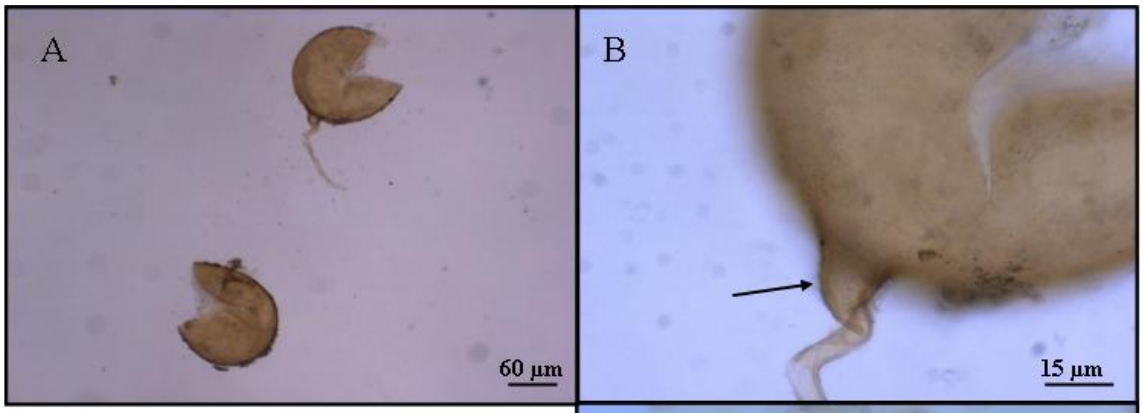


Annex 1.1: Schematic representation of the roots and soil sample strategy.



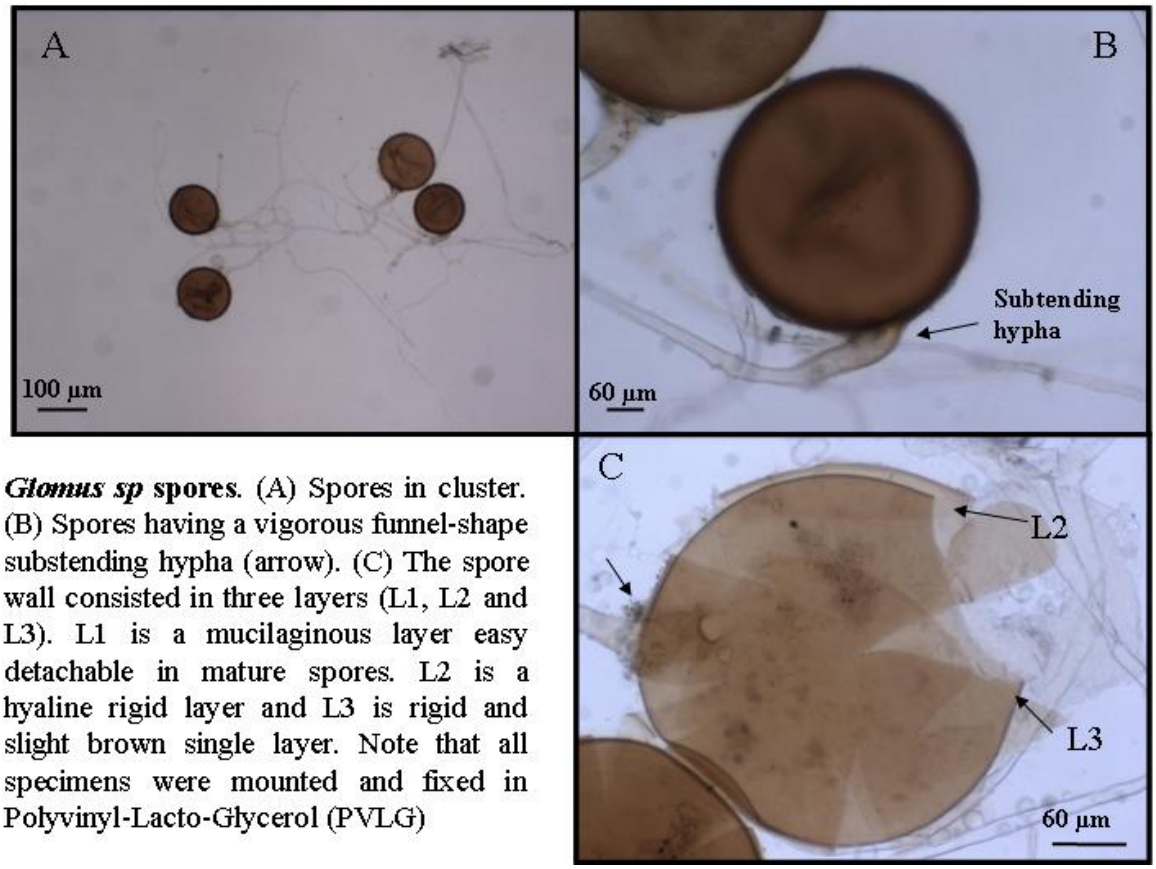
Annex 1.2: *In vitro* culture initiation from spores or colonized roots

(a) Inoculum production from trap cultures. (b) Choice of starter inoculum and isolation process: Spores and mycorrhizal roots are collected from the trap cultures by wet sieving. Spores and roots are examined under dissecting microscope and recovered. Root pieces are used only if they contain sufficient vesicles. (c) Disinfection process: Several disinfecting agents are used: ethanol, calcium hypochlorite, chloramine T added with some drops of Tween 20 and antibiotics (streptomycin and gentamycin), (for details see Cranenbrouck et al., 2005). (d) Incubation process: After disinfection, the propagules are incubated on a synthetic growth medium until hyphal re-growth is observed. (e) Association process: Germinating spores and mycorrhizal root pieces showing hyphal re-growth are associated with root-organ cultures or *Medicago truncatula* plantlets.



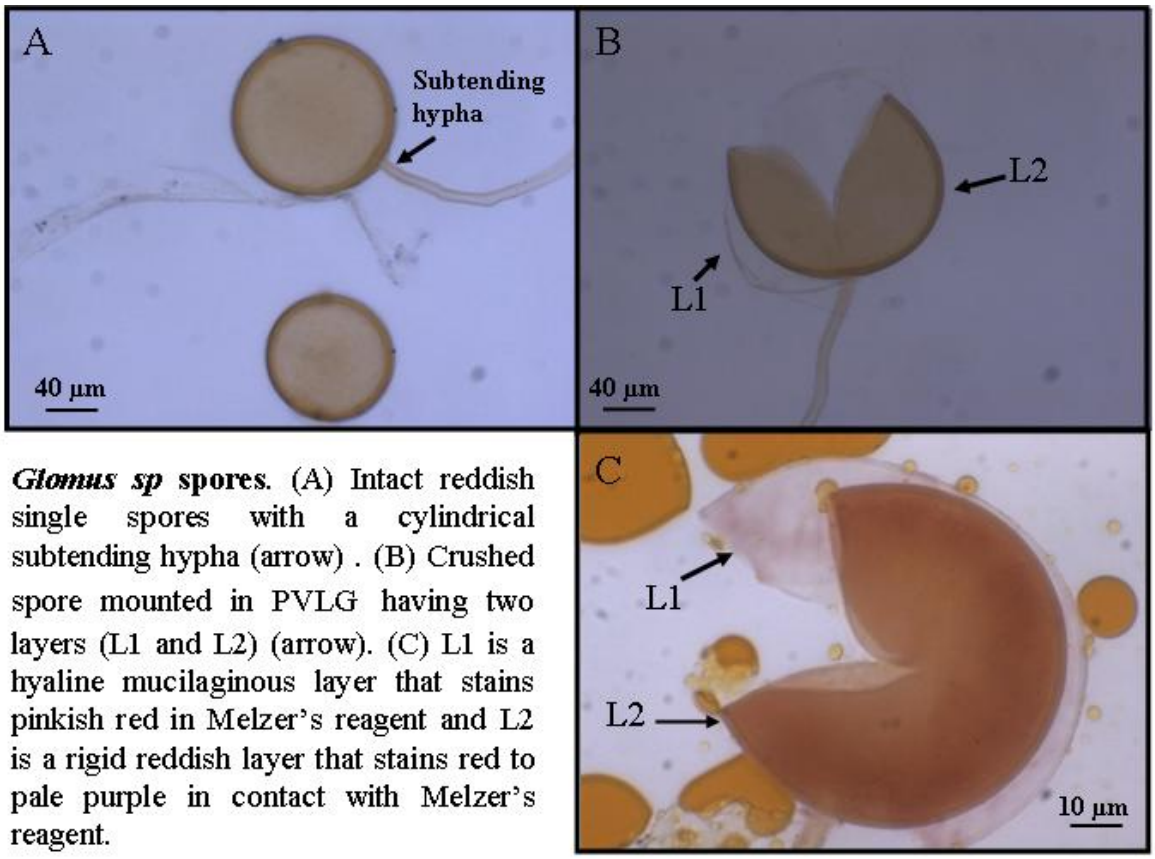
***Glomus mosseae* spores**. (A) Single-Crushed yellow-brown spores. (B) Robust funnel-shaped subtending hypha (arrow). (C) The spore wall consisted in three layers (L1, L2 and L3) (arrow). **L1** is a hyaline, mucilaginous and easily detached layer, rarely present in mature spores. **L2** is a hyaline and generally rigid layer and fracturing into sliver-like fragments. **L3** is a laminate yellow-brown layer. Note that all the specimens were mounted fixed in Polyvinyl-Lacto-Glycerol (PVLG).

Annex 1.3: Morphological description of *G. mosseae* spores.



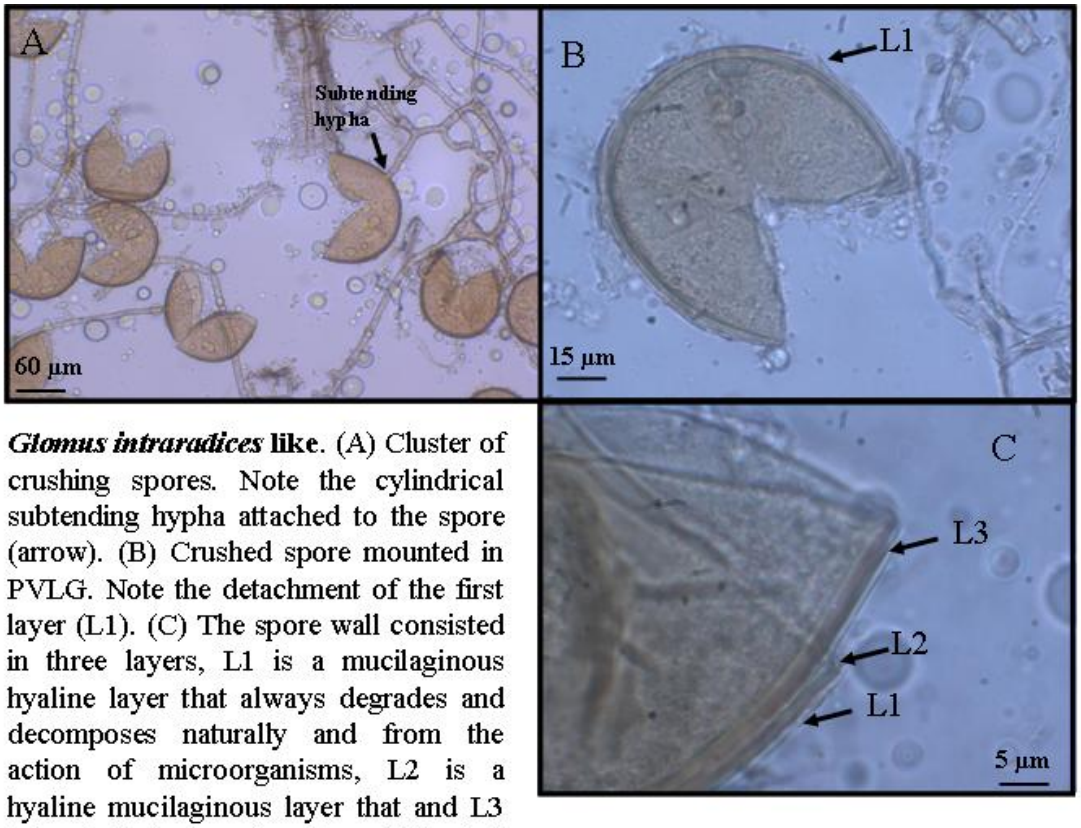
***Glomus sp* spores**. (A) Spores in cluster. (B) Spores having a vigorous funnel-shape substending hypha (arrow). (C) The spore wall consisted in three layers (L1, L2 and L3). L1 is a mucilaginous layer easy detachable in mature spores. L2 is a hyaline rigid layer and L3 is rigid and slight brown single layer. Note that all specimens were mounted and fixed in Polyvinyl-Lacto-Glycerol (PVLG)

Annex 1.4: Morphological description of *Glomus sp* spores.



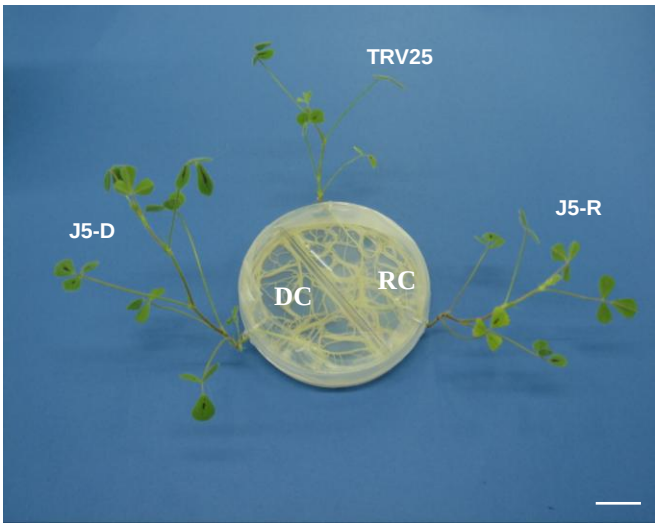
***Glomus sp* spores**. (A) Intact reddish single spores with a cylindrical subtending hypha (arrow) . (B) Crushed spore mounted in PVLG having two layers (L1 and L2) (arrow). (C) L1 is a hyaline mucilaginous layer that stains pinkish red in Melzer's reagent and L2 is a rigid reddish layer that stains red to pale purple in contact with Melzer's reagent.

Annex 1.5: Morphological description of *Glomus sp* spores.



***Glomus intraradices* like**. (A) Cluster of crushing spores. Note the cylindrical subtending hypha attached to the spore (arrow). (B) Crushed spore mounted in PVLG. Note the detachment of the first layer (L1). (C) The spore wall consisted in three layers, L1 is a mucilaginous hyaline layer that always degrades and decomposes naturally and from the action of microorganisms, L2 is a hyaline mucilaginous layer that and L3 is laminated pale yellow layer. Note that spores are mounted in PVLG reagent.

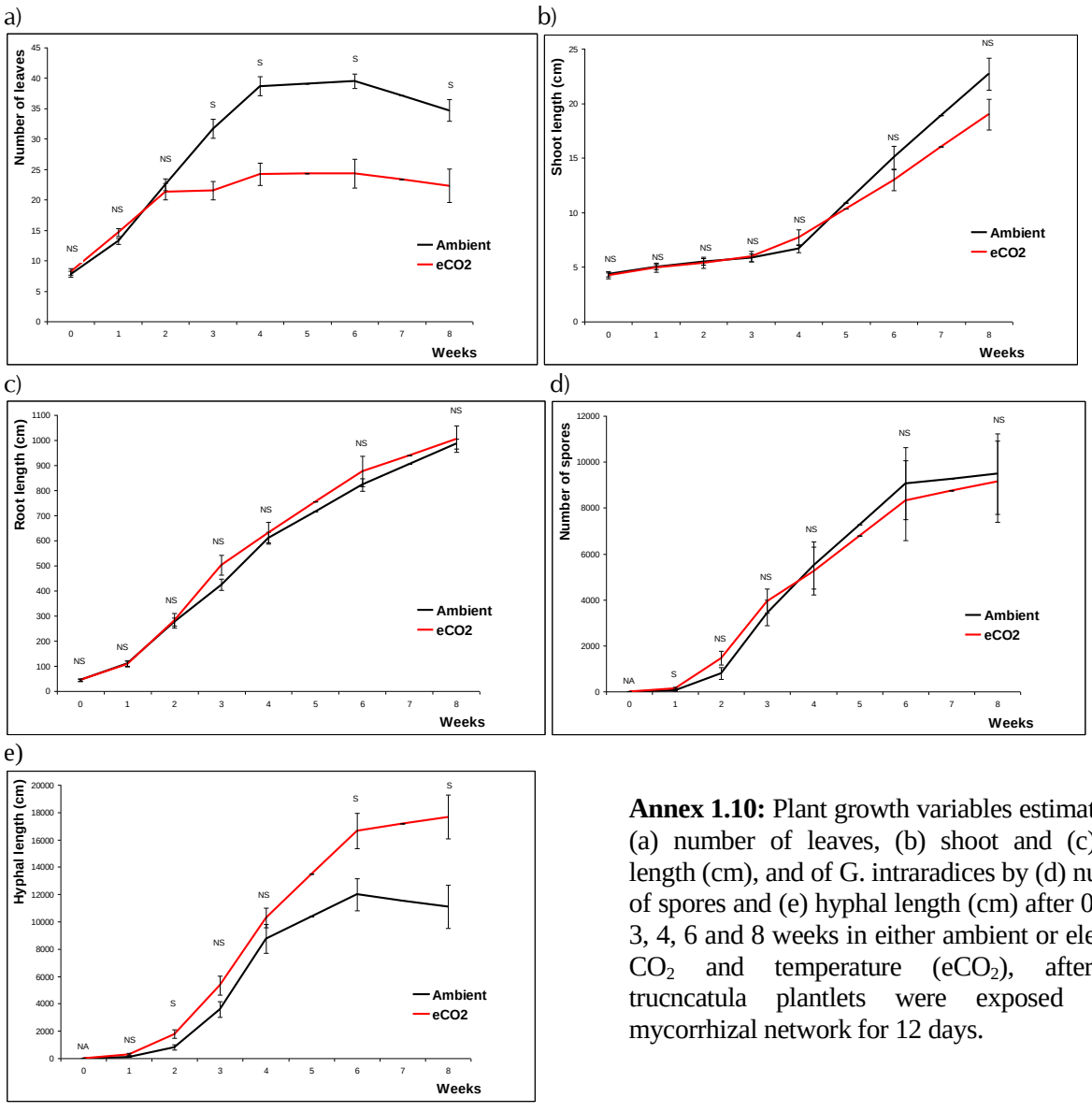
Annex 1.6: Morphological description of *G. intraradices*-like.



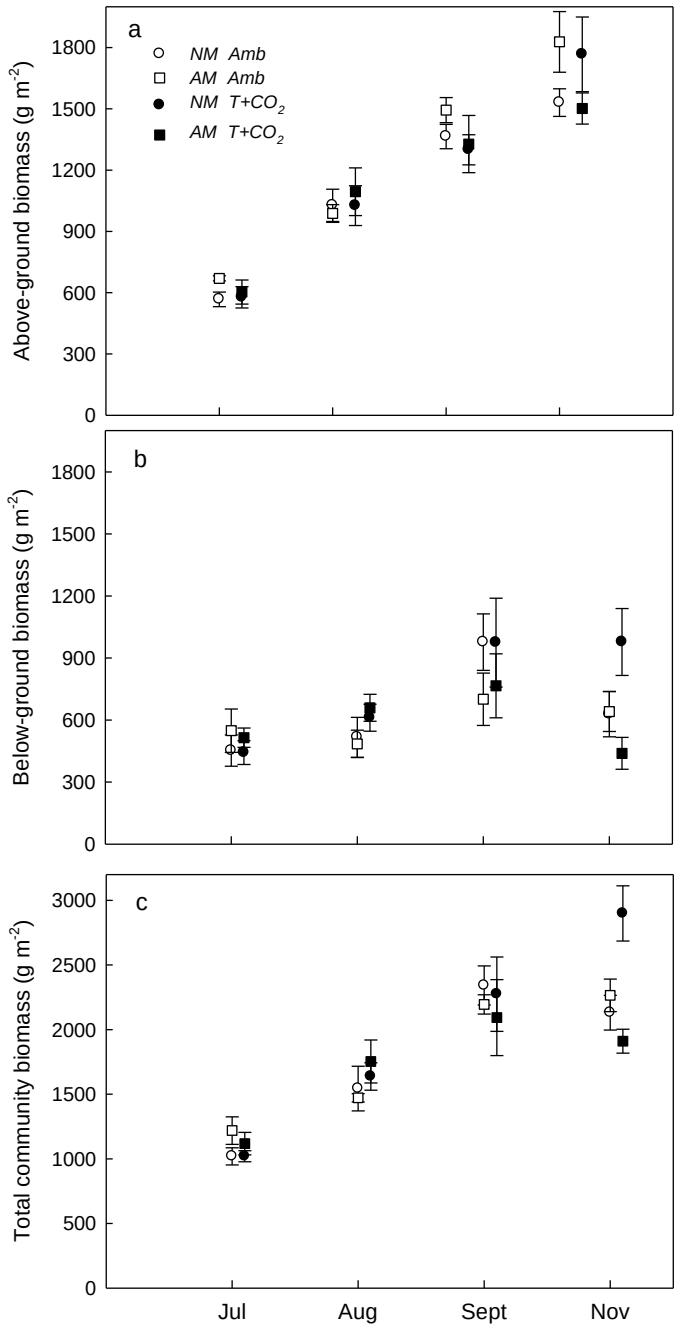
Annex 1.7: Representation of the *in vitro* experimental system, developed to investigate carbon transfer from a donor (J5-D) to a receiver (J5-R) *Medicago truncatula* plant linked by a common extraradical network of an arbuscular mycorrhizal fungus (AMF), *Glomus intraradices*. The two plants developed in separated compartments (a donor compartment – DC and a receiver compartment – RC) physically separated by a plastic wall, while the AMF crossed the wall and interconnected the two plants. A non-mycotrophic plant of M. truncatula (TRV 25) was introduced in the RC, as control. Scale bar = 2 cm.

Annex 1.8: Representation of the *in vitro* experimental system, developed to investigate carbon transfer from *Medicago truncatula* (J5) to *Centaurium erythrae*a, linked by a common extraradical network of an arbuscular mycorrhizal fungus (AMF), *Glomus intraradices*. The two plants developed in separated compartments (a donor compartment – DC and a receiver compartment – RC) physically separated by a plastic wall, while the AMF crossed the wall and interconnected the two plants. A Myc-/Nod- plant of *M. truncatula* (TRV 25) was introduced in the RC, as control. Scale bar = 2 cm.

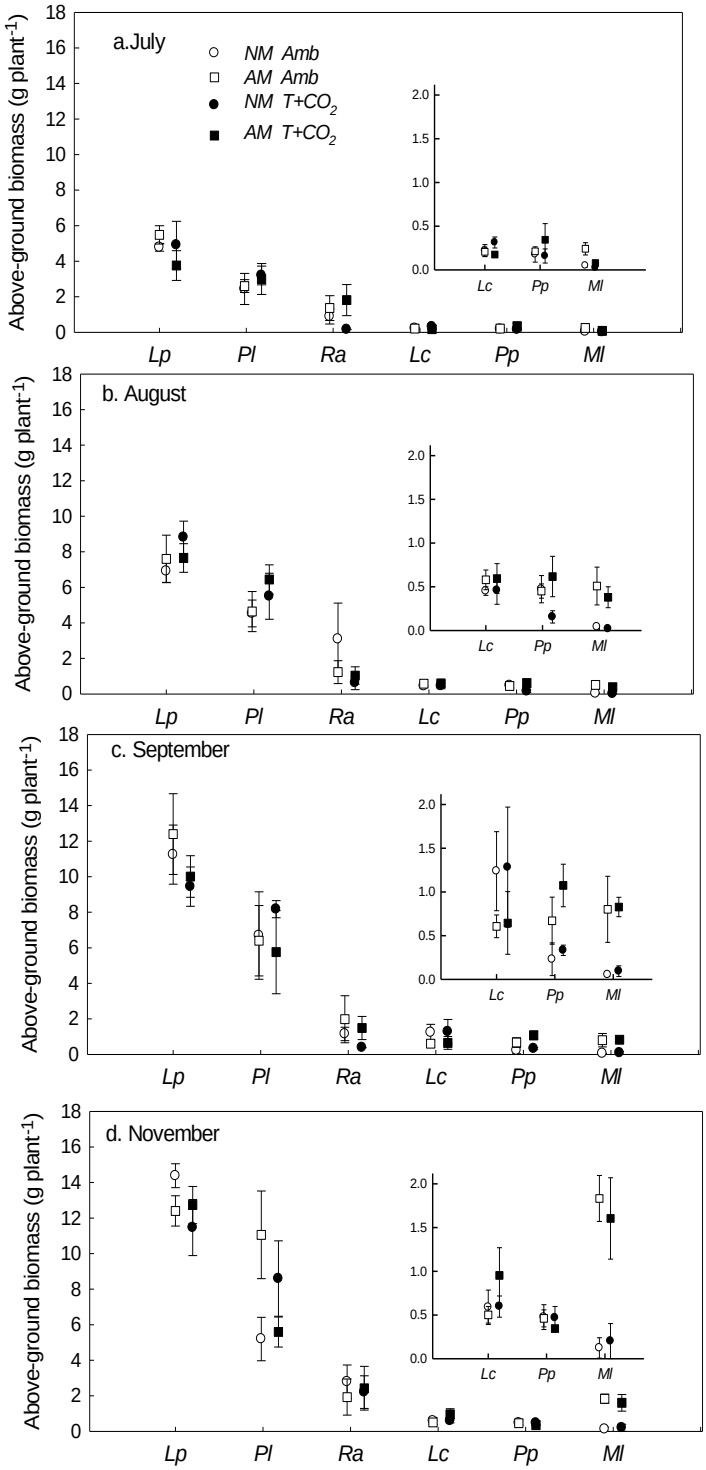
Annex 1.9: Climate-controlled chambers of the grassland experimental platform at the Drie Eiken Campus in Wilrijk, University of Antwerp (left), and inside view of a chamber with various grassland communities (right). The chambers are used to simulate future climate scenarios with elevated temperature and CO_2 concentration and enable studies at the community level on the effects of climatic changes on above- and below-ground components of grasslands.



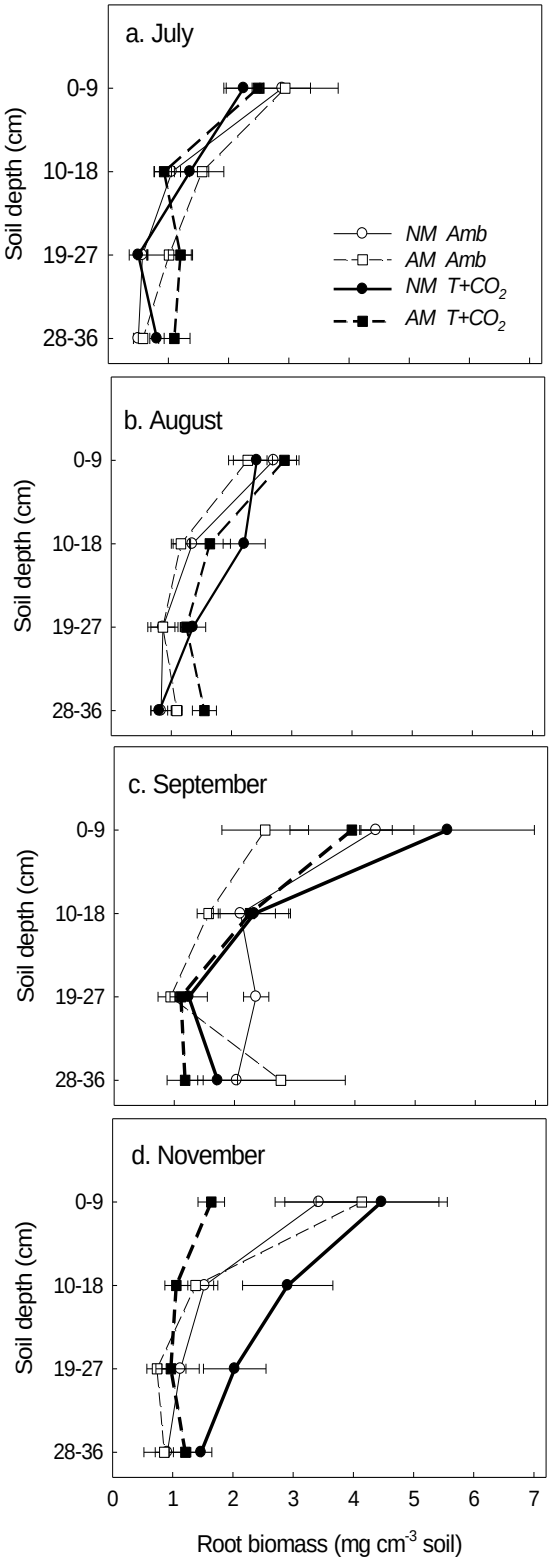
Annex 1.10: Plant growth variables estimated by (a) number of leaves, (b) shoot and (c) root length (cm), and of G. intraradices by (d) number of spores and (e) hyphal length (cm) after 0, 1, 2, 3, 4, 6 and 8 weeks in either ambient or elevated CO_2 and temperature (eCO_2), after M. trucncatula plantlets were exposed to a mycorrhizal network for 12 days.



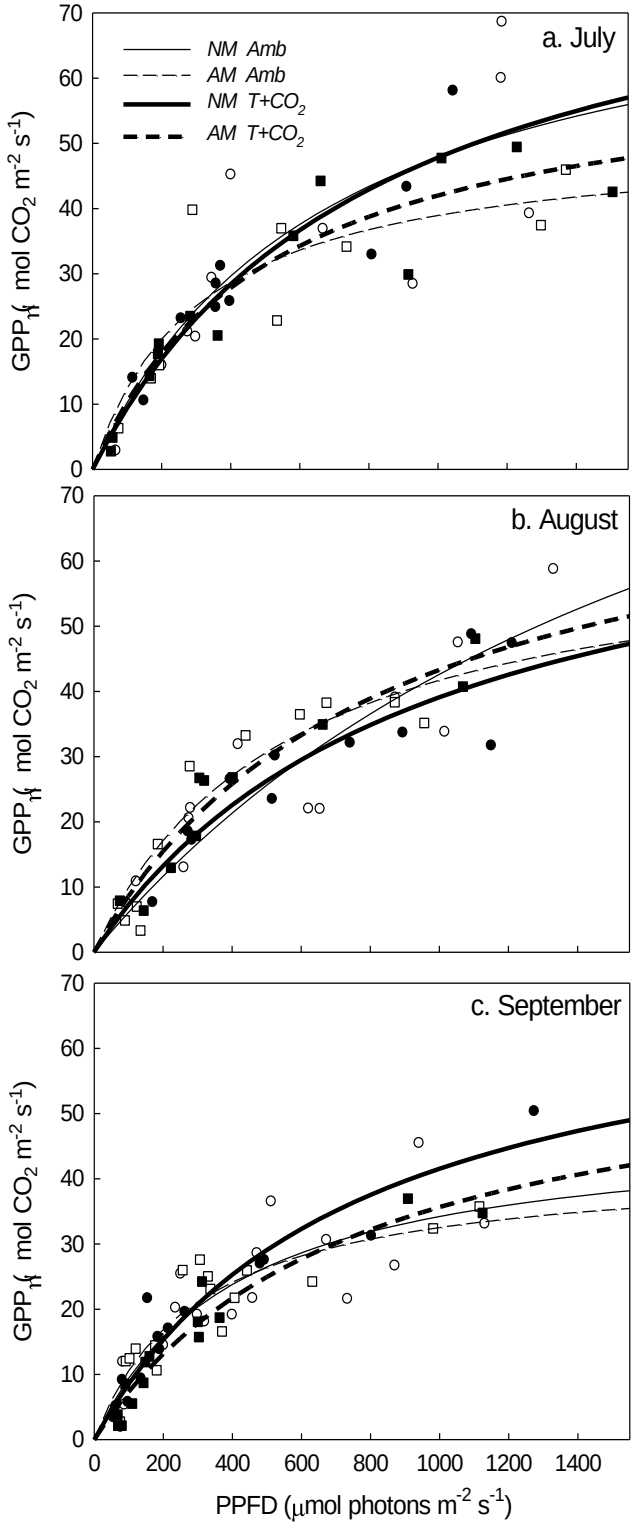
Annex 1.11. Time course of (a) above-ground biomass, (b) below-ground biomass, and (c) total community biomass of grasslands exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO$_2$,*) grown in the presence (*AM*) or absence (*NM*) of arbuscular mycorrhizal fungi. Means ± SE of 3 replicates in July (day of the year, DOY 201), August (DOY 230) and September (DOY 267). In November (DOY 310) means ± SE of 6 replicates for above-ground biomass and 4 replicates for below-ground and total community biomass.



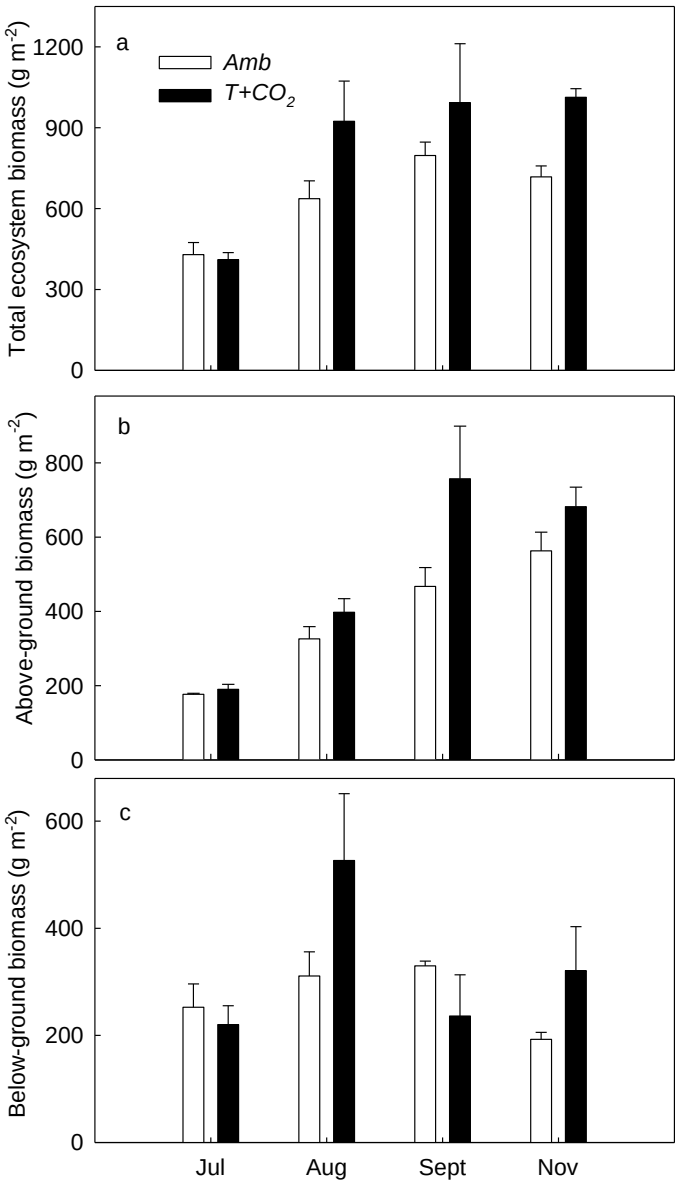
Annex 1.12: Above-ground biomass of individual species during different periods of the growing season of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO₂*), grown in the presence (*AM*) or absence (*NM*) of arbuscular mycorrhizal fungi. Means ± SE of 3 replicates in July (a, day of the year, DOY 201), August (b, DOY 230), and September (c, DOY 267), and of 6 replicates in November (d, DOY 310). Species: *Lolium perenne* (*Lp*), *Plantago lanceolata* (*Pl*), *Rumex acetosa* (*Ra*), *Lotus corniculatus* (*Lc*), *Poa pratensis* (*Pp*), *Medicago lupulina* (*Ml*). Insert: magnification of species with low biomass.



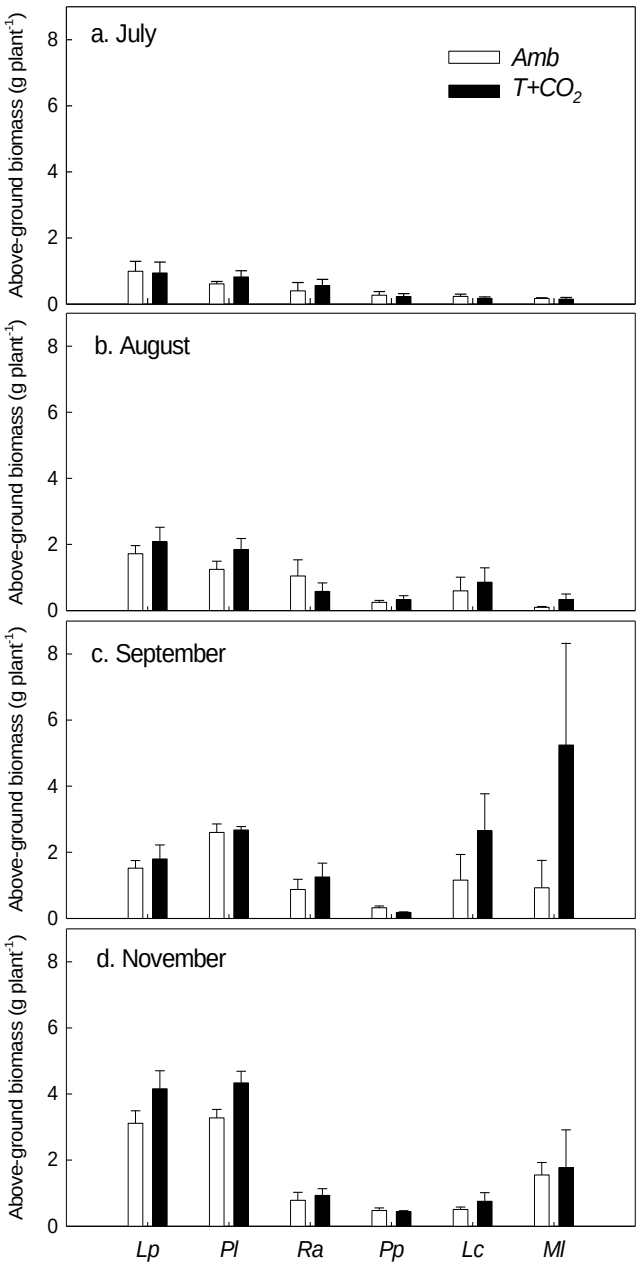
Annex 1.13. Below-ground biomass in four soil layers during different periods of the growing season of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO2,*), grown in the presence (*AM*) or absence (*NM*) of arbuscular mycorrhizal fungi. Means ± SE of 3 replicates July (a, day of the year, DOY 201), August (b, DOY 230), and September (c, DOY 267), and of 4 replicates in November (d, DOY 310).



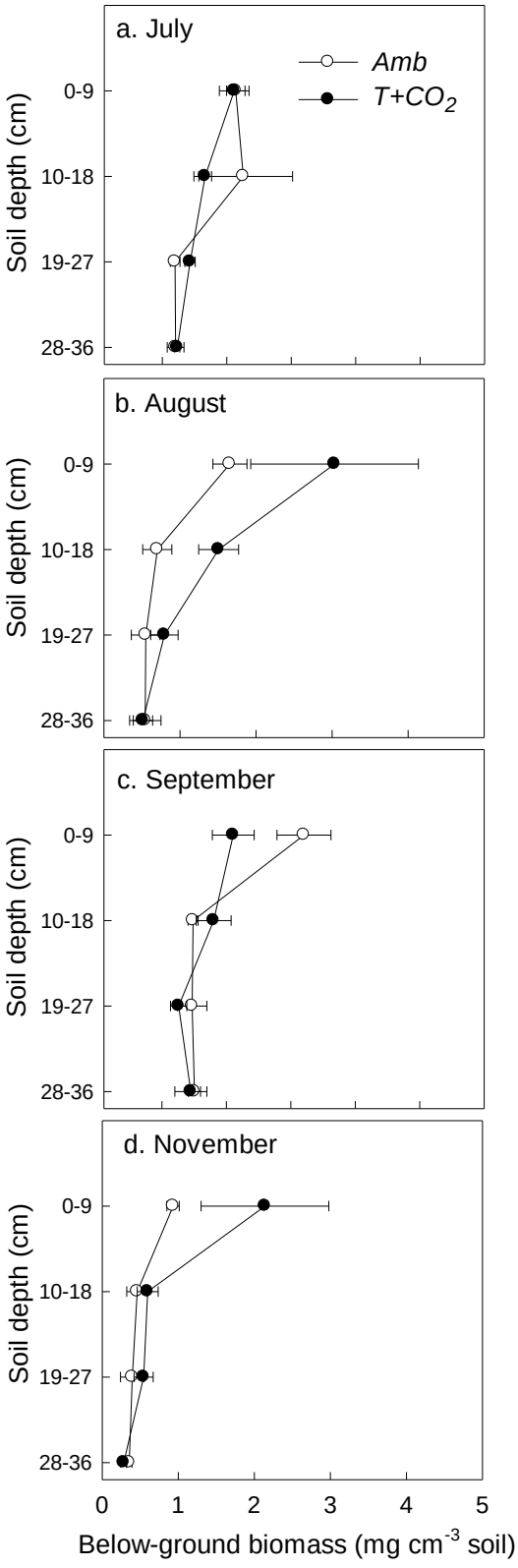
Annex 1.14. Fitted regression on the relationship between gross primary productivity (GPP) and photosynthetic photon flux density (PPFD) in (a) July, (b) August, and (c) September of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO₂*) grown in the presence (*AM*, squares) or absence (*NM*, circles) of arbuscular mycorrhizal fungi. Fitted regression lines are given separately for *AM* (solid lines) and *NM* (dashed lines).



Annex 1.15. Time course of (a) total community biomass, (b) above-ground biomass, and (c) below-ground biomass of grasslands exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO2,*). Means ± SE of 3 replicates in July (day of the year, DOY 201), August (DOY 230) and September (DOY 267). In November (DOY 310) means ± SE of 6 and 4 replicates for above- and below-ground biomass, respectively.

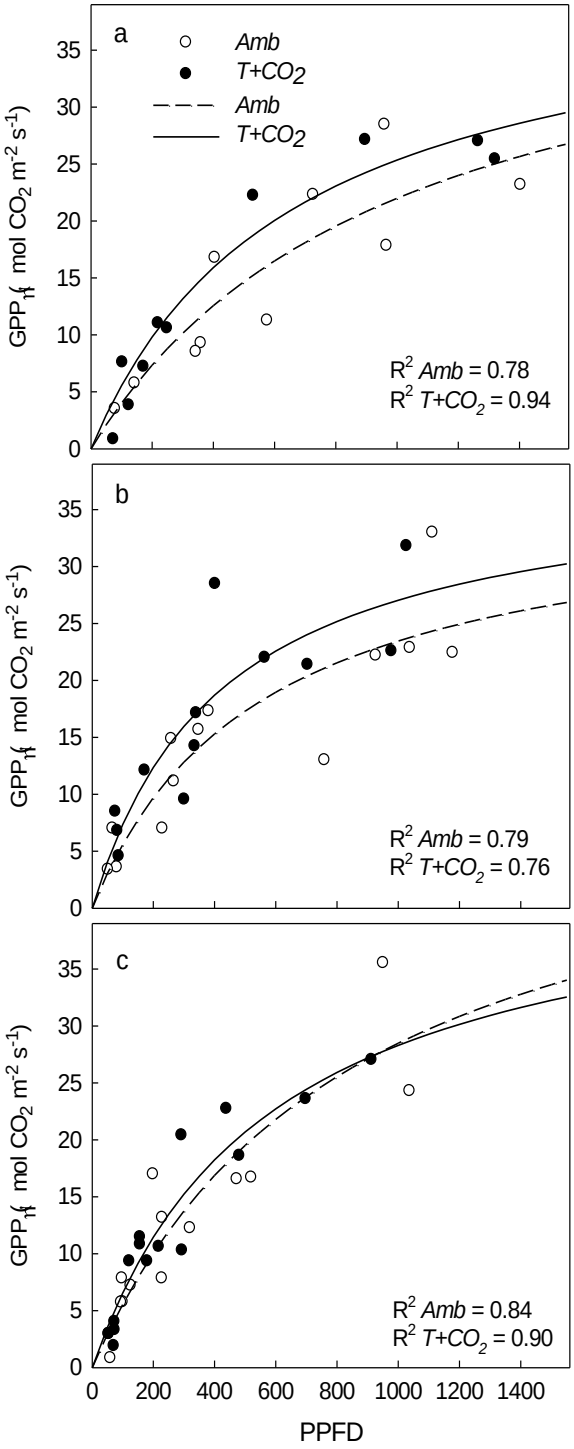


Annexe 1.16. Above-ground biomass of individual species at different periods of the growing season of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO2*). Means ± SE of 3 replicates in July (a, day of the year, DOY 201), August (b, DOY 230), and September (c, DOY 267), and of 6 replicates in November (d, DOY 310). Species: *Lolium perenne* (*Lp*), *Plantago lanceolata* (*Pl*), *Rumex acetosa* (*Ra*), *Lotus corniculatus* (*Lc*), *Poa pratensis* (*Pp*), *Medicago lupulina* (*Ml*).

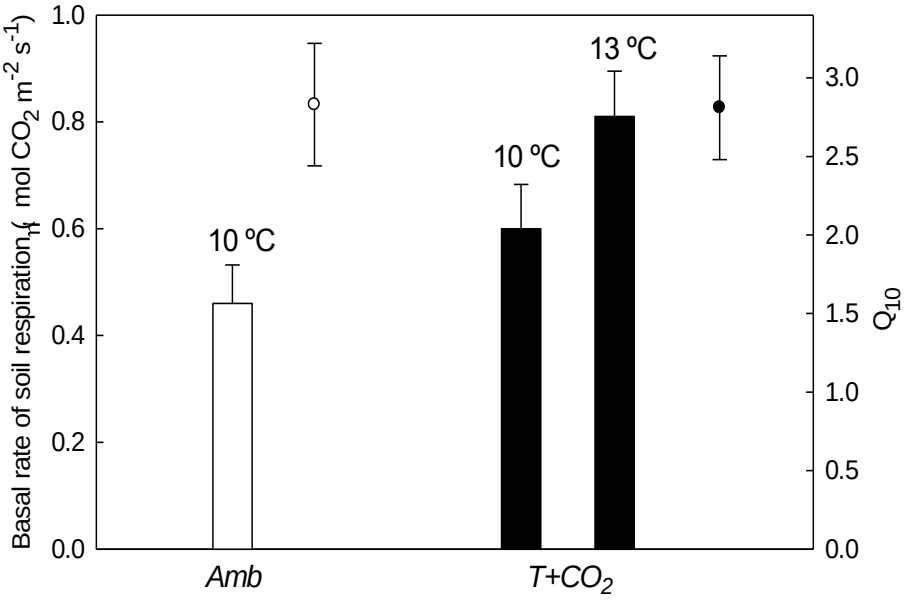


Annex 1.17: Below-ground biomass at four soil layers during different periods of the growing season of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient

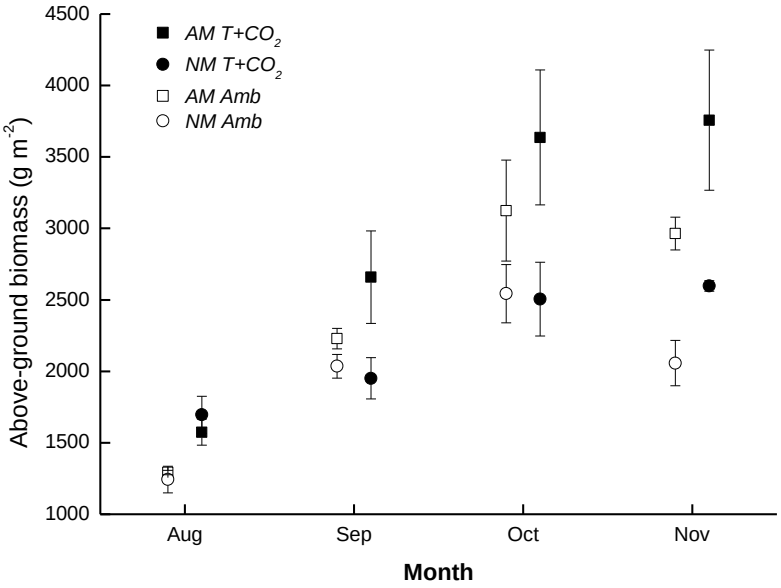
temperature +3 ºC and 620 ppm of CO_2 ($T+CO_2$). Means ± SE of 3 replicates July (a, day of the year, DOY 201), August (b, DOY 230), and September (c, DOY 267), and of 4 replicates in November (d, DOY 310).



Annexe 1.18: Fitted regression curves to the relationship of gross primary productivity (GPP) and photosynthetic photon flux density (PPFD) in (a) July, (b) August, and (c) September of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 ($T+CO_2$).



Annex 1.19: Weighted mean basal rate of soil respiration and temperature sensitivity (Q_{10}) of grassland communities exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature +3 ºC and 620 ppm of CO_2 (*T+CO₂*) measured at the end of the season. Circles represent the Q_{10} (white and black circle for *Amb* and the *T+CO₂*, respectively). Basal rate of soil respiration was computed at growth temperature of 10 °C and 13 °C for the *Amb* (white bar) and the *T+CO₂* (black bars) scenario. For the *T+CO₂* scenario, the basal rate of soil respiration is also shown at a reference temperature of 10 °C. Weighted means ± SE of 5 replicates.



Annex 1.20: Time course of above-ground biomass of grasslands exposed to ambient temperature and 375 ppm of CO_2 (*Amb*) or ambient temperature + 3°C and 620 ppm of CO_2 (*T+CO₂*) grown in presence (*AM*) or absence (*NM*) of arbuscular mycorrhizal fungi. Means ± SE of 5 replicates in August (day of the year, DOY 228), September (DOY 253), October (DOY 281) and November (311).