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ABSTRACT

Climate change is expected to affect terrestrial ecosystems across the globe with increased atmospheric CO_2 concentration, higher temperatures and changes in the precipitation patterns. These environmental factors are drivers of many important ecosystem processes, and changes in ecosystem function are therefore expected in the future.

Research into climate change effects has focussed much on the aboveground effects, while belowground effects are less studied. Further the responses of fine roots to climate change have mostly been studied in experiments manipulating single factors, while little attention has focussed on how such factors will interact and affect root system size and dynamics. The background for this thesis was to investigate the effects of elevated CO_2, increased temperature and summer drought and their interactions on root dynamics and nutrient uptake in a Danish heathland.

The standing root biomass and root length was positively affected by elevated CO_2 in all combinations for the two studied species *Calluna vulgaris* (heather) and *Deschampsia flexuosa* (wavy hair-grass). This resulted in more roots deeper in the soil, which can add new carbon to deeper soil layers but might also prime turnover of old soil organic matter and alter the carbon balance. Mining for nutrients is probably the main reason for greater root proliferation in deeper soils. When nutrient uptake was studied, the two plant species did not increase their uptake of nitrogen (N) in response to elevated CO_2, and as a result of the higher growth the root N concentration was decreased, mostly in *Deschampsia*. Phosphor (P) concentration was not affected by the treatments, but in *Calluna* roots warming seemed to alleviate the P demand. Warming generally had a positive effect on *Calluna* root biomass, while warming and drought in combination had a negative effect on *Deschampsia* root biomass. Warming also decreased the root length observed in minirhizotrons and this might be a result of lower soil water content, higher mineralization and higher turnover in the upper part of the soil. The belowground plant nitrogen N and P pool were increased in the full treatment combination mimicking our future climate, which indicates that in this ecosystem root growth is not yet strongly nutrient limited, after 5 years of climate treatments.

Throughout the study many interactions of the treatments were found, and our research underscores the value of long term, multi factorial experiments.

RESUME

Det forventes, at klimaændringerne vil påvirke økosystemer globalt med højere atmosfæriske CO_2-koncentrationer, varmere klima og ændringer i nedbørsmønsteret. Disse miljøfaktorer er drivkraft bag mange økologiske processer, og der forventes fremover at ske ændringer i økosystemers funktion.

Forskning inden for klimaændringer har længe fokuseret på de overjordiske processer, mens der ikke er lavet så mange undersøgelser under jorden. Derudover har de fleste klimaforsøg kun fokuseret på at variere en enkelt klimafaktor, og der er derfor ikke megen viden om, hvordan ændrede klimafaktorer vil interagere og dermed påvirke planterødder.
Baggrunden for denne ph.d.-afhandling er at undersøge, hvordan forhøjet CO_2, opvarmning og sommertørke i samspil vil påvirke rod-dynamikken og næringsoptaget på en dansk hede.

Rodbiomasse og rodlængde var positivt påvirket af højere CO_2-koncentrationer i alle behandlingskombinationer for de dominerende planter, Hedelyng og Bølget Bunke. Dette resulterede i flere dybe rødder, hvilket kan tilføre ekstra kulstof dybere nede i jorden, men også kan øge nedbrydning af 'gammel kulstof' ved at stimulere nedbrydningen og derved ændre kulstofbalancen. Dybe rødder kan afsøge et større jordvolumen for næringsstoffer og er en måde at øge næringsoptaget på. Ved at undersøge optaget af kvælstof (N) i rødderne fandt vi, at planterne ikke øgede N-optaget i samme grad, som de voksede. Dette medførte en lavere koncentration af N i rødderne, særligt i Bølget Bunke. Rodkoncentrationerne af fosfor (P) var ikke ændret i klimabehandlingerne, men fosfor-optaget viste, at der var lavere mangel af P i hedelyng, når den voksede under opvarmning.

Opvarmning havde generelt en positiv effekt på Hedelyngs rodbiomasse, mens særligt opvarmning i kombination med tørke påvirkede rødderne i Bølget Bunke negativt. Observation af rødder i jorden via minirhizotroner viste, at antallet af rødder også blev lavere i tørkebehandlingen. Denne negative effekt blev opvejet, når tørke blev kombineret med forhøjet CO_2. Opvarmning havde også en negativ effekt på rodlængderne i de øverste jordlag og kan skyldes lavere jordvandskoncentrationer, højere mineralisering og højere omsætningshastighed i jorden. Plantepuljerne af N og P i jorden øgedes i den behandling, der efterligner vores fremtidige klima i Danmark. Dette tyder på, at rodvæksten ikke er stærkt næringsstofbegrænset efter 5 års klimabehandlinger.

Hele vejen igennem forsøget opstod der signifikante interaktioner mellem behandlingerne, hvilket understreger vigtigheden i at lave længerevarende forsøg med flere faktorer, der kan interagere med hinanden.

PREFACE AND ACKNOWLEDGEMENTS

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LIST OF PAPERS

I.   Arndal MF, Schmidt IK, Tolver A, Beier, C. Changes in fine root growth and rooting pattern to elevated CO_2, warming and drought in a mixed heathland-grassland.
Manuscript to be submitted to Global Change Biology.

II.   Arndal MF, Schmidt IK, Kongstad J, Beier C, Michelsen A. Fine root growth and N dynamics in responses to climate change: results from the CLIMAITE experiment.
Submitted to Oecologia, Nov. 2011.

III.   Arndal, MF, Merrild MP, Michelsen A, Schmidt IK, Beier C. Root growth and nutrient acquisition in response to future climate change in two heath land plants.
Manuscript to be submitted to New Phytologist.

THE AIM OF THE PROJECT

The objectives of this PhD was to examine below-ground root production, standing biomass, root distribution, input of root litter to the soil and root litter decay rate in response to a Danish climate change scenario anno 2075.

The specific research questions raised were:

- How does root biomass change in response to elevated CO_2, warming and drought?
- Does the vertical distribution of roots change in response to climate change?
- Are there changes in root lifespan and root turnover in response to climate change?
- Does root nutrient uptake match an expected increased growth, under elevated CO_2?
- Is root litter decomposition changed in a future climate?

The answers to these questions are addressed in the three papers I, II and III, and in the introductory part. The introductory part gives a brief overview of the study and the theory behind, with a discussion of methods and the results. Following the introduction is a methodological appendix and finally three papers, one submitted and two manuscripts ready for submission.

GENERAL INTRODUCTION

Climate change

Anthropogenic combustion of fossil fuels have increased the amount of CO_2 in the atmosphere, and the amount in 2005 (379 ppm) exceeds by far the natural range of the last 650,000 years (IPCC, 2007). This increase is mainly due to fossil fuel and land use changes, and is expected to increase even further depending on the level of future increase in CO_2 emissions. Due to CO_2 being a green-house gas and absorbing long-wave energy, the atmosphere is warming. In the last 100 years this has caused about a 0.74 °C increase in global average temperature, and eleven of the twelve years in the period 1995–2006 rank among the top 12 warmest years in the instrumental record (since 1880) (IPCC, 2007). Furthermore, increases of both drought and heavy precipitation events are also expected globally.

In Denmark the temperature are expected to increase by 2-3 $^{\circ}$C in 2100 compared to 1990. Night temperature is expected to increase more than day temperature and winter temperature will increase relatively more than summer temperature. Precipitation is expected to increase in winter time, while in the summer time precipitation will decrease, with risk of drought periods. Extreme precipitation events are expected, especially in autumn (Danish Meteorological Institute, http://www.DMI.dk).

The CLIMAITE experiment

The CLIMAITE manipulation experiment started in October 2005, with the purpose of studying climate change effects on biological processes in terrestrial ecosystems. The experiment was made as cooperation between Danish Universities (University of Copenhagen, University of Aarhus and Technical University of Denmark, former Risø).

The manipulations in the CLIMAITE experiment are designed according to the climate predictions for Denmark in year 2075 as predicted by the Danish Meteorological Institute. There is however one important exception: precipitation is forecasted to change with prolonged summer droughts and increased winter precipitation, but with no major changes in annual amounts. The CLIMAITE experiment focussed on the summer drought only, because potential responses would be difficult to interpret in a combined summer removal and winter addition scenario.
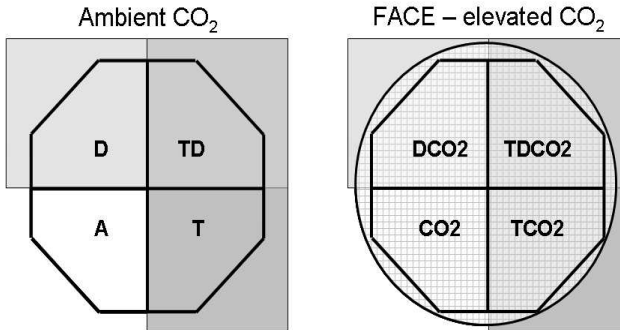
The climate scenario used in the experiment is elevated atmospheric CO_2 concentration at 510 ppm, elevated temperatures of 1-2 °C and a prolonged summer drought period (4-6 weeks). The experiment consists of 12 octagons (7 m in diameter) laid out pair wise in 6 blocks in a Danish heathland. Each block consists of two octagons with one octagon receiving elevated CO_2 (CO_2, 510 ppm) by FACE technique and the other receiving ambient CO_2 (A). Within each octagon there are four subplots with the following treatments: summer drought (D, exclusion of rain by automatic shelters), elevated temperature (T, passive night time warming by reflective curtains), a combination of drought and elevated temperature (TD), and an untreated control for reference (A) in a split plot design. The experiment provides a full factorial design replicated 6 times with the treatments and combinations: A, T, D, CO2, TD, TCO2 DCO2 and TDCO2, giving a total of 48 plots. The treatments were initiated in October 2005. See Mikkelsen *et al.* (2008) and Larsen *et al*. (2011) for further details on experimental design and set up, and see Fig. 1 and Table 1 for 'CLIMAITE' data.

Aerial photo of a Danish heathland where the CLIMAITE experiment is situated. At the photo, both warming and drought curtains are displayed (photo by Kim Pilegaard).
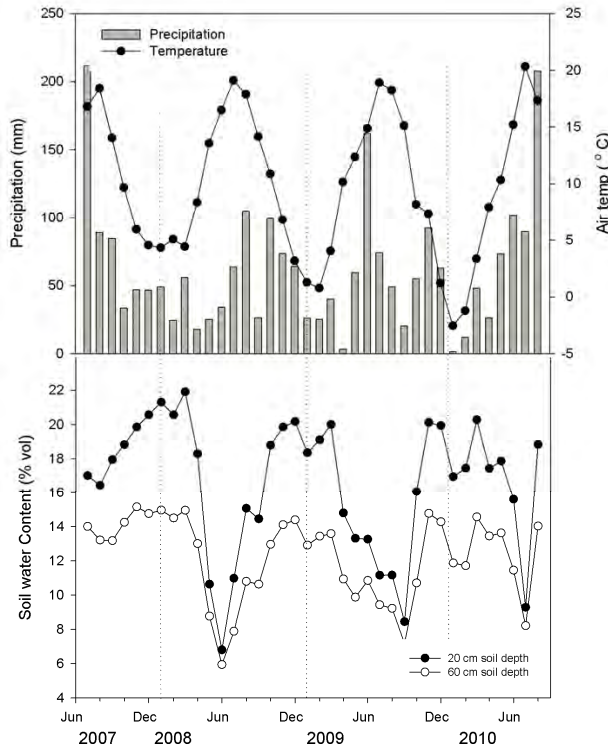
Fig 1. Average precipitation (mm), air temperature (°C) and soil water content (vol %) in the study period (2007-2010) in control plots (treatment A).

Table 1. *CLIMAITE data showing the dates for drought periods, amount of water excluded during drought in % of annual precipitation, and soil water content (SWC) measured by TDR probes in 0-20 cm depth (Vol%).*

	A	D	T	TD	CO2	DCO2	TCO2	TDCO2
2007 Drought period: 21/5- 22/6								
Prec. excl %.	-	11	-	11	-	11	-	11
SWC in 20 cm	17.31	15.63	15.96	14.88	16.37	16.81	15.72	14.96
2008 Drought period 5/5- 27/5 and 16/9- 2/10								
Prec. excl %.	-	6	-	6	-	6	-	6
2009 Drought period:18/5 -25/5 and 25/6 – 13/7								
Prec. excl %.	-	3 and 4	-	3 and 4	-	3 and 4	-	3 and 4
SWC in 20 cm	15.47	13.86	13.71	12.90	14.02	14.41	13.87	13.29
2010 Drought period: 4/5- 3/6								
Prec. excl %.	-	9	-	9	-	9	-	9
SWC in 20 cm	17.43	14.83	15.55	14.61	15.38	17.40	16.29	16.25

Plant roots and the importance of studying roots

The earliest land plants had no roots, but when first roots had evolved, large terrestrial biomass on land could develop and reducing soil erosion (Harper *et al.* 1991). The root systems in the soil anchored large plants and allowed for uptake of water and nutrients, and linked the visible part of plants with the soil system (Gregory, 2006).

The roots support a temporally and spatially heterogeneous microbial community (Farrar *et al.* 2003), by exudation of sugars and amino acids that provide labile C and N to microorganisms. The soils are important habitats for fungi, invertebrates, bacteria etc, and especially in the small volume of soil surrounding the root, called the rhizosphere (Hinsinger *et al.* 2009).

Roots of higher plants provide much of the carbon to the soil compartment in ecosystem, in which resources are frequently scarce and patchy and with great variation down the soil profile (Hinsinger *et al.* 2009). Depending on species and developmental stage of plants, as much as 25-50% of carbon assimilated per day are allocated to roots, where approximately half of that is used in respiration (Marschner, 1995). Some of the photosynthates are used in rhizodeposition, i.e. carbon compounds released from living roots to the rhizosphere. In annual species photosynthetic carbon allocated to rhizodeposition may account for 4-70%, (Marschner, 1995). The C allocated belowground can have a number of fates in the short term: 1) conversion into root biomass, 2) respiration for new growth, ion uptake and maintenance 3) re-export to shoots, 4) diversion to symbionts such as mycorrhizae, 5) loss as exudates, or sloughed cells, 6) loss to herbivory, 7) loss in the process of turnover (reviewed by Pritchard and Rogers, 2000). Addition of rhizodeposits to the soil can increase the decomposition rate and has important implications for nutrient dynamics (Marschner, 1995). By root exudation the plant may regulate the soil microbial community and change the chemical and physical properties of the soil.

Root growth is often larger than aboveground production in grass lands (Mokany *et al.* 2006) and heath lands (Johansson, 2000; Aerts and Heil, 1993) and in light of climate change and ongoing debate on C sequestration by the vegetation, the role of roots have become increasingly important. It has been suggested that a large proportion of the additional carbon is being sequestered in terrestrial vegetation, and that much of that is stored belowground (Gifford *et al.* 1996), also reported for temperate grasslands (Soussana and Luescher, 2007). Thus carbon cycling and the ability to sequester C will be driven more by belowground than aboveground processes. Hence climate models, which are predicting future changes to climate, are

dependent on belowground field studies to give a realistic representation of root processes and dynamics. As much as 33% of global annually NPP is used for fine root production (Jackson *et al.* 1997), and if total NPP are estimated from satellite observations alone, this would result in erroneous C fluxes through vegetation (Woodward and Osborne, 2000). There is thus a need to achieve more knowledge on the coupling between climatic change and ecosystem C-cycling belowground in order to better understand and predict possible feed-back mechanisms of the global carbon balance.

Root response to climate change - current knowledge

The effects of climate change on roots are difficult to predict (Eissenstat *et al.* 2000). Climate change may affect a number of root parameters, as for example root biomass, which is important when determining the vegetation carbon stocks, and the root production and turnover, which determines the flux of C to the soil through C and N cycling. Also root length might be affected, which is important when determining the capacity for nutrient uptake.

CO_2 RESPONSE

Elevated CO_2 has been shown to increase carbon assimilation in plants (Albert *et al.* 2011a; Albert *et al.* 2011; de Graaff *et al.* 2006). Part of this C will be transported belowground and soil C sequestration is expected to be enhanced under elevated CO_2. It has been suggested that elevated CO_2 can stimulate root growth or root activity and provide a positive feedback on plant growth.

Plant growth has been shown to increase under elevated CO_2 (Rogers *et al.* 1994) with more carbon being allocated to the roots (VanVuuren *et al.* 1997; Fitter *et al.* 1997) but negative responses of CO_2 are also reported (Arnone *et al.* 2000; Higgins *et al.* 2002).

Elevated CO_2 was also assumed to increase the root:shoot ratio in earlier studies, but new studies have revealed less pronounced effects (Bielenberg and Bassirirad, 2005). Poorter and Nagel (2000) concluded in their review that there would be no significant changes in response to a doubling of CO_2 concentrations. They also highlighted the importance of other environmental factors that would regulate the plant biomass allocation response to elevated CO_2. The Progressive Nitrogen Limitation (PNL) hypothesis suggests that without additional N input or reduced N loss, the N availability decreases over time at elevated CO_2 (Luo *et al.* 2004), leading to reduced plant growth and C uptake in the long term. Hence there is still much debate on whether higher CO_2 stimulates root biomass allocation, and studies are needed to de-

termine if increased root growth under elevated CO_2 conditions results in sequestration of carbon below ground.

Nutrient uptake kinetics is expected to change under elevated CO_2. This can be due to higher availability of carbohydrates under elevated CO_2 which may result in up-regulation of root nutrient transporters. Also elevated CO_2 accelerates growth and hence increase plant nutrient demand and uptake capacity (Bielenberg and Bassirirad, 2005). Nevertheless most studies of root nutrient uptake in response to CO_2 enrichment have produced highly inconsistent patterns (Bielenberg and Bassirirad, 2005), which may result from differences in protocols, but species dependent responses have also been reported by Bassirirad *et al.* (2001).

However, it is not yet known whether root growth and physiological properties can adjust to meet an increased nutrient demand in a future climate with higher CO_2 concentrations (Bassirirad *et al.* 1997). Reduced plant N concentrations are often observed under elevated CO_2 (Taub and Wang, 2008; Gifford *et al.* 2000; Gill *et al.* 2006), and an increase in plant C:N ratio potentially cause decrease in litter quality, which determines the decomposition (Prescott, 2005), and hence the root turnover. Therefore factors that affect the availability and uptake of nutrients are also critical in determining plant and ecosystems responses to elevated CO_2.

WARMING RESPONSE

The reports on root response to warming are not consistent. There have been reports of no effect of warming on root production (Dukes *et al.* 2005; Hollister and Flaherty, 2010) and negative effects (De Boeck *et al.* 2007; Lilley *et al.* 2001). However, warming may also stimulate root growth up to a maximum temperature (Gregory, 2006), probably due to increases in photosynthesis with higher temperatures, where the extra fixed carbon is allocated belowground to sustain new root growth (Pregitzer and King, 2005). However, in a review of 85 warming studies, warming enhanced the aboveground biomass but did not significantly affect the belowground biomass (Wu *et al.* 2011), probably as the indirect effect of higher temperatures are more complex, as almost all chemical and biological processes are impacted by temperature (Shaver *et al.* 2000).

Besides root growth soil temperature also affects root physiology in several ways by altering the specific rates of ion uptake, root respiration, cell membrane permeability etc. Nutrient uptake capacity generally increases in response to increasing temperatures – although caution should be made about generalization across a broad range of species and soil temperatures (Bassirirad, 2000).

Generally data suggests that the indirect effects of warming are more important than the direct effects. One indirect effect may be longer growing

season in response to warming (Prieto *et al.* 2009) and higher mineralization (Rustad *et al.* 2001; Schmidt *et al.* 2002). The main factors that determine decomposition and thus the availability of nutrients are water and temperature together with litter quality. Hence root turnover has been reported to increase exponentially with mean annual temperature for fine roots of grasslands (Gill and Jackson, 2000).

When high soil temperatures are associated with drought conditions, which are predicted in the future, there is no reason to expect an increase in root growth (Pregitzer *et al.* 2000).

DROUGHT RESPONSE

Several studies show that drought decreases the rate of root length extension (reviewed by Pregitzer *et al*, 2000), as reduced water availability reduces the turgor pressure within the root and therefore reduces growth rate (Davies and Bacon, 2003). Decreased precipitation suppressed aboveground biomass in all 85 studies, but there was insufficient data available to assess the effects on belowground biomass, in the review by Wu *et al.* (2011).

Drought is affecting the functioning of the thinner roots and the diameter can also be affected due to increased mechanical impedance in drying soils (Ostonen *et al.* 2007). Low soil moisture reduces nutrient availability and nutrient uptake as it prevents mass flow and diffusion of nutrients in the rooting zone (Gutschick and Pushnik, 2005; Pregitzer and King, 2005). Nutrient deprived plants up-regulate the ion uptake, and when nutrient supply is restored after a drought period, a high root uptake capacity for nutrients might allow the plant to optimize the ion capture in competition with other species (Glass, 2005). Higher CO_2 concentrations might alleviate the drought effect, due to the increased water use efficiency of plants.

INTERACTIONS

Although there have been many studies of root dynamics and response to CO_2, there are few studies of the effects of interactions among CO_2, temperature and soil moisture (but see Garten *et al.* 2009; Shaw *et al.* 2002). The largest emphasis has been on elevated CO_2 alone, due to the presumed direct feedback between root turnover responses to elevated CO_2 and the cycling of CO_2 through ecosystems and back to the atmosphere (Norby and Jackson, 2000). General responses to changes in temperature and precipitation and their combined effects are still not well understood, and the interactive effects of warming and altered precipitation tended to be lower than the expected from the single-factor responses (Wu *et al.* 2011).

The increased water use efficiency under elevated CO_2 and the increased evatransporation under elevated temperature might offset each other and together have an intermediate effect on root dynamics (Johnson *et al.* 2006).

Water limitation is often shown to amplify the percentage response of plant growth to elevated CO_2, caused by the reduced transpiration under elevated CO_2 leading to improved soil–water status (McMurtrie *et al.* 2008). However, these interactions have to be studied in multi factorial experiments.

HEATH LANDS

The inland dry heathlands are semi natural ecosystems that are nutrient poor and dominated by the heather *Calluna vulgaris* (L.) Hull. As a result of human activities, such as N deposition, fragmentation, lack of management and climate change (Aerts and Heil, 1993) heathlands have declined dramatically in most countries during the last centuries and nowadays account for 7 % of the European land cover (http://www.eea.europa.eu).

The most characteristic plant species of nutrient poor dry heathlands in Denmark are the heather, *Calluna vulgaris*, but concurrently with the lately threats and changes in the dry heathlands, the wavy-hair grass, *Deschampsia flexuosa* (L.) Trin has increased in abundance within this ecosystem. Since the species have very different growth forms and live strategies, the change from dwarf shrubs to grasses has consequences for both biodiversity and ecosystem function.

Calluna is an evergreen perennial dwarf shrub, which seldom exceeds 1.25 m in height (Gimingham, 1960). Root biomass is almost entirely confined to the upper 10 cm of the soil profile (Aerts and Heil, 1993). The lateral roots are extensively suberized after the first year. Fine roots are non-suberized, brown and without root hairs. Young plants have a well marked tap root, which is later obscured by stronger growth and branching of laterals (Heath and Luckwill, 1938). *Calluna* roots had a maximum rooting depth of 40 cm in a study in heathlands in The Netherlands.

Deschampsia is a perennial grass, and as there are always green leaves present, *Deschampsia* has been characterized as a 'semi-evergreen' (Aerts and Heil, 1993). The rhizomes are branched underground, must abundant in the humus layer. Roots reach depths of at least 58 cm, but probably much deeper (Scurfield, 1954).

However, the observations from our experimental site indicated that both *Deschampsia* and suberized *Calluna* roots grew down to app. 50-70 cm soil depth, estimated from soil cores. The deeper root distribution could probably be due to this site being drier.

MYCORRHIZAS

The word 'Mycorrhiza' means an association between fungi and root, and mycorrhizal fungi play a major role in nutrient uptake by land plants and are a key component of ecosystem carbon and nutrient cycling (Smith and Read, 2008). In nutrient limited ecosystems such as heathlands, mycorrhizal sym-

bioses play an important role in nutrient uptake of the dominant species. *Deschampsia* roots are colonized by arbuscular mycorhizal (AM) fungi, which are known to enhance plant nutrient uptake, especially of phosphorus (Smith and Read, 2008). Roots of *Calluna* are likely to be colonized by ericoid mycorrhizal (ErM) fungi. ErM fungi are capable of improving host plant nutrient uptake since they can access organic nitrogen by production of extracellular enzymes (Näsholm *et al.* 1998), thus improving access to nitrogen sources which are not accessible to other competing, non-ericaceous plant roots (Michelsen *et al.* 1996).

Global change factors will affect most ecosystems and plants; hence their associated mycorrhizal fungi are also likely to be affected. AM fungi have been found to respond positively to increased atmospheric CO_2 concentrations (Treseder, 2004), and warming has also been reported to cause positive AM fungal responses (Rillig *et al.* 2002; Staddon *et al.* 2004). Prolonged summer drought increased the proportion of root length colonized while decreasing the density of external mycelial hyphae (Staddon *et al.* 2003). In a subarctic forest ecosystem it has previously been found that ErM colonization increased under elevated atmospheric CO_2 concentrations (Olsrud *et al.* 2010). Understanding of mycorrhizal dynamics is important in order to understand carbon and nutrient cycling, but as we still lack basic knowledge on mycorrhizal fungi, predictions of global change effects of the fungi are difficult (Fitter *et al.* 2004). To provide a complete picture of belowground processes and the coupling of fungi to the overall responses of ecosystems to climate change, investigation of mycorrhizal fungi are required.

METHODOLOGY

The life of roots takes place below ground and thus the roots are invisible from above, unlike leaves. Root studies therefore often involve destructive sampling and tedious sorting of samples, which makes it impossible to study the root dynamics *in situ*. So far no single technique has proved capable of providing all the necessary information needed to study roots in soils, so a variety of methods have been used to collect measurements of different parts of the system (Gregory, 2006), as in this study.

The methods used in this PhD study were: soil coring, in-growth cores, minirhizotrons and litter bags, and later nutrient uptake and the use of isotopes became a part of the study. Due to the limited space and expensive set-up of the CLIMAITE experiment, non-destructive root studies were prioritized. All methods are briefly described and discussed here, but see the three papers for further information on method and execution of study.

SEQUENTIAL SOIL CORING

A common approach of determining fine root biomass in the field is the sequential soil coring method (Vogt *et al.* 1998). Roots collected from soil cores may give information on mass, diameter, length and nutrient concentration but not on longevity (Mackie-Dawson and Atkinson, 1991). Sequential soil coring can be used for studying biomass and how roots are distributed with depth and changes over time, but this requires many destructive samplings. Hence in a long term experiment like CLIMAITE, it is not possible to take up soil cores several times throughout the year, as it simply disturbs the site too much.

We estimated the standing root biomass from 4 destructive harvests spread out over 1.5 years. The soil cores were mainly taken with a soil corer of 4.5 or 5 cm in diameter. Separating roots from the soil samples can be done by washing, but as soon as there is any debris it needs to be hand picked by forceps, making it a laborious job. All the root sorting was therefore done by hand, due the large amounts of litter and organic material in our soil samples.

Distinguishing live from dead roots is difficult and the criteria are usually based on colour and physical appearance, but time consuming staining and/or microscopic examination has also been used (Gregory, 2006). We did not separate living and dead roots, only if there was clear visual difference indicating a dead root.

In-growth cores are root free soil contained in a mesh bag that is incubated in a drilled hole in the soil. Plant roots can easily grow through the mesh into the new soil volume. After a predetermined time, the mesh bags are removed in order to measure the amount of roots that has grown into the bags. Our in-growth cores were established under *Calluna* and *Deschampsia* vegetation respectively in each of the 48 plots. Soil was extracted from soil cores taken in the experimental plots (5 cm in diameter) and after sieving of the soil, the soil was filled up in a mesh bag (2 mm mesh size) and the meshbags/ingrowth cores were reinstalled under the two species. The original soil cores taken in mixed vegetation (March 2007) and under *Calluna* and *Deschampsia* (April 2007) were used for estimating the standing crop of root, and the soil without roots was then put in the in-growth cores. The following two in-growth cores used the same soil and original holes in the soil, to minimize the damage of destructive soil coring at the site. The age of the different in-growth cores were 6 months, 1 year and 1.5 years.

The in-growth cores can be a good alternative to soil coring for studying seasonal dynamics (Steen, 1991). However, this requires many cores to be collected in short intervals during a growing season, which was not possible at the site. Some criticism of the method might be an overestimation the root production, as roots may proliferate more due to 1) no root competition, 2) higher nutrient availability following soil disturbance, 3) as a response to coring (damaging of roots), 4) and due to changes in the soil bulk density in the in-growth core (reviwed by Joslin and Wolfe, 1999). However, as a measure for relative responses to experimentally induced climate changes, the method may give valid results.

We did not separate living and dead roots, but as the long turnover time from minirhizotron suggest the amounts of dead roots in in-growth cores of max. 1.5 years are probably small.

The roots from the in-growth cores were also seen to be very suitable for nutrient assays, as all roots experienced the same conditions and had the same maximum age.

MINIRHIZOTRONS

Minirhizotrons are transparent acrylic tubes installed in the soil, and are a non destructive method where birth and death of individual roots can be followed through time with a camera or scanner. The use of minirhizotrons makes it possible to measure root diameter, length, branching, orientation and root hairs on individual roots, and by repeated measurements it is possible to follow roots for several years and estimate longevity. At each sampling date we determined the total standing root length pr tube by summing all root lengths (mm) present in a window during a sampling date. As much time in this PhD was spent on minirhizotron methodology, the discussion and comment on this method is presented separately in Appendix I.

Schematic drawing of minirhizotron installed in soil (left hand side, © CID Inc.), and a subsample of a minirhizotron image (right hand side) showing roots growing outside the minirhizotron tube (sub sample image with area 1.5 x 3 cm).

ROOT LITTERBAG STUDIES

In the soil roots are exposed to a different environment of heterotrophic organisms than aboveground tissues, and changes in precipitation and temperatures might have great impact on these organisms and their decomposition of roots. Root litterbag studies can be used for understanding the fine-root decay processes. To take the effect of litter changes into account, it is necessary to incubate litter produced under climate manipulations. However, it was impossible to get root litter in our experimental plots due to the disturbance, and we therefore used litter collected outside the experimental area, and thus are not able to evaluate the effect of changes in litter quality to climate change but solely the direct effect of climate on decomposition.

The litterbags used in the experiment were made of nylon fabric (gardisette) with mesh size of approximately 1 mm. In each experimental plot under both *Calluna* and *Deschampsia* plants, 4 litterbags were buried under the litter layer as close to the soil surface as possible. The first litter bags were taken up after 6 months, and the following after 1, 1.5 and 2.75 years.

The rapid disappearance of fine roots observed in many minirhizotron studies (Stewart and Frank, 2008; Johnson *et al.* 2001) suggest that fine root decomposition might be underestimated by measuring the mass loss in litterbag studies (Hendrick and Pregitzer, 1996). One reason is that when collecting roots for litterbags, the finest roots, which decompose fast, are probably lost during the litterbag sample preparation (Hendrick and Pregitzer, 1996). However, our results are used for studying the relative differences between treatments on decomposition. One way of improving the method would be to use some of the newly collected root material from sequential coring at the site, as these roots probably have differences in chemistry due to the climate treatments, and might result in changes in composition that are more realistic.

ROOT TURNOVER AND LIFESPAN

Turnover is defined as the 'replacement of a particular standing stock' (Lauenroth and Gill, 2003). Root turnover is an important parameter to measure, due to the great effect on the C balance. Turnover can be estimated by several methods, based on net primary production, root length turnover from minirhizotrons or direct estimates based on ^{14}C turnover (Lauenroth and Gill, 2003).

We estimated root turnover from minirhizotrons according to Milchunas *et al* (2005): new length growth summed over periods of one year divided by total root length averaged across periods for that year (Milchunas *et al.* 2005). We calculated turnover from the last year of measurement, i.e. from July 2009 to July 2010.

Fine root lifespan can be estimated by several methods, but the use of minirhizotrons and carbon isotopes are widely used. However, the two methods differ, leading to an overestimate of turnover by direct observations (minirhizotrons) and underestimate by the use of isotopic techniques (Guo *et al.* 2008; Strand *et al.* 2008; Trumbore and Gaudinski, 2003). Sah *et al.* (2011) concluded that ^{14}C signature of fine roots may not always be indicative of root age, either because of addition of C from unknown sources such as storage reserves, or some other processes.

We did not measure the lifespan of fine roots, as insufficient time had elapsed for a survival analysis to be carried out.

EXCISED ROOTS ASSAY

The response to climate change is much dependent on the nutrient availability and nutrient uptake of plant roots, which is likely to change in the future. Changes in the nutrient uptake might influence the ability of ecosystems to sequester excess C and there is still lacking information of root nutrient uptake in a future climate (Bassirirad, 2000).

One way of determining plant nutrient limitation is by bioassays, where excised roots are placed in a solution with the isotopically labelled nutrient to be studied, and afterwards determine the uptake (Jones *et al.* 1991).

The first bioassay in this study was done with roots of *Calluna* and *Deschampsia* using NO_3^--N and NH_4^+-N in October 2008, while the second assay was done with NH_4^+-N and PO_4^{3-} in the summer 2010. In most plants, root kinetics of N uptake is regulated by demand which is likely to exhibit a seasonal pattern. Seasonal activities of soil organisms produce substantial changes in the availability of required nutrients (Glass, 2005), and the two assays represent two different seasons.

The nutrient assays were done on excised roots from in-growth cores, as a way of studying the root nutrient demand in the climate treatments and on fairly even aged roots (Paper II and III). Bassirirad (2000) has shown that root excision decreases nutrient absorption of NH_4^+-N and NO_3^-N already 2 hours after the roots were detached from the plant. Also, the excision effect (compared to intact roots) was substantial on *Deschampsia* roots in a study of inorganic nitrogen uptake (Falkengren-Grerup *et al.* 2000) and in barley plants (Bloom and Caldwell, 1988). However, it was not possible to study intact plants in this study, but the excised root method has earlier been used to quantify nutrient limitation (Harrison and Helliwell, 1979; Jones *et al.* 1991; Michelsen *et al.* 1999), and was a tool to get more knowledge on the root nutrient demand in the different treatments at our site. We were not interested in the true uptake rate of nutrients, but more interested in the relative differences between the treatments, to conclude on the future nutrient demands.

MYCORRHIZAS

Changed root uptake kinetic responses to elevated CO_2 is just one potential mechanism to increase the nutrient uptake, and adjustments in other root characteristics might prevent the need for changes in nutrient uptake kinetics as suggested by Bielenberg and Bassirirad (2005). They recommended that future studies with climate change should, among other things, pay more attention to mycorrhizal associations, when determining the plant nutrient response. Mycorrhizas affect the nutrient uptake of plant roots and hence in order to study root nutrient uptake responses to global change, the amount of mycorrhiza was included in our study of nutrient uptake (Paper III).

Roots of both *Calluna* and *Deschampsia* were visually examined to determine plant species and fungal colonization of ericoid (ErM) and Arbuscular (AM) mycorrhiza, Dark septate endophytes (DSE) and Fine endophytes (FE) in response to the climate treatments.

It has been questioned whether percentage root colonization is the best way to measure mycorrhizal responses to elevated CO_2. One of the main dis-

advantages is that this method does not distinguish live and dead hyphae. Other concerns are 1) percent root colonization does not necessarily correlate with nutrient transfer to the plant, 2) the increase in root colonization might not be as big as the response to fungal biomass and 3) the root colonization may not respond to elevated CO_2 while the intensity, frequency or abundance of arbuscules and vesicles may change significantly (reviewed by Cavagnaro *et al.* 2011). However, this method is still widely used, especially as other methods might be very time consuming.

RESULTS

Many of the results are presented in Paper I-III and are therefore only sum-
marized in the results section below. Some of my data, which are not pre-
sented in the papers, are shown in graphs or tables in the section below.
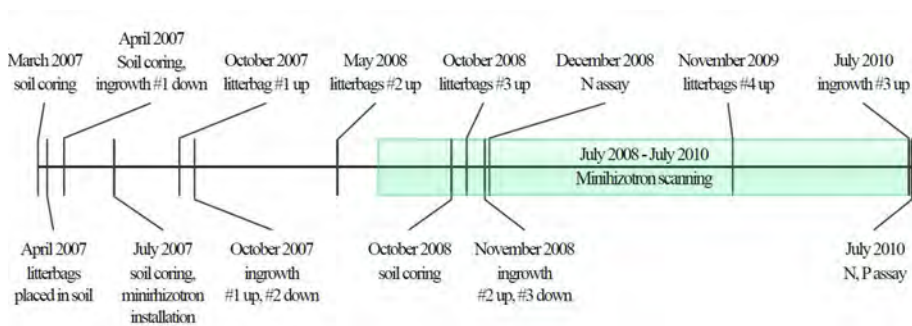
Fig. 2. *Timeline for field and lab work during the study period (2007-2010).*

Standing crop root biomass

The standing root crop showed an expected seasonal pattern with the highest
biomass measured for the mixed vegetation in July, and the lowest biomass
of both species from autumn and early spring (Table 2). The biomass for
Calluna was also high in April, suggesting the growing season had begun for
this evergreen species.
In 2007, two years after treatments began, no significant treatment effects
were found when looking at the whole soil profile, while in 2008 both spe-
cies and all soil depths responded to the treatments (see Paper II and III).
This also confirms the need for longer term experiments, as slow growing
ecosystems might not respond to the treatments in the first years.

Table 2. *Standing crop root biomass (g m^{-2}, mean±1SE) from four soil samplings under either mixed vegetation of Calluna and Deschampsia or taken separately under each species, in O-horizon, 0-5 cm or 5-10 cm, 10-30 and 30-70 cm soil depth. The treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations TD, TCO2, DCO2 and TDCO2.*

	A	T	D	TD	CO2	TCO2	DCO2	TDCO2
Mixed vegetation, March 2007								
O-hor	70±15	62±8	79±16	68±22	110±54	88±16	97±23	50±9
0 til 5	117±25	119±11	159±27	148±31	156±16	137±19	178±19	159±13
5 til 10	71±28	55±5	64±7	61±9	87±15	60±8	77±10	75±6
10 til 30	47±17	40±6	59±12	58±10	54±10	52±9	51±10	58±7
Calluna, April 2007								
Ohor	119±56	78±23	84±21	60±20	95±46	47±22	64±15	138±70
cal 0-5	204±41	183±30	208±41	199±30	285±61	240±44	241±33	211±38
cal 5-10	100±14	104±10	69±7	75±10	94±20	128±21	107±15	118±23
Deschampsia, April 2007								
Ohor	74±26	58±9	98±34	95±29	91±26	99±30	81±16	73±11
des 0-5	161±37	168±26	137±25	134±30	177±31	251±44	186±35	151±17
des 5-10	65±14	117±19	68±11	71±6	124±31	99±15	68±9	101±26
Mixed vegetation, July 2007								
Ohor	63±23	84±37	101±28	59±23	73±23	187±58	136±35	53±15
0 til 5	245±52	167±29	242±43	148±20	225±34	185±24	179±44	194±17
5 til 10	120±15	82±17	101±18	79±9	105±11	132±23	107±26	113±12
10 til 30	123±28	77±13	80±13	90±32	85±7	76±14	90±18	115±16
30 til 70	46±17	36±5	55±15	46±17	94±29	50±18	76±19	68±23
Calluna, Oct. 2008								
Ohor	89±17	74±14	106±47	69±18	136±57	78±16	106±20	64±19
0 til 5	173±33	106±13	145±27	146±23	98±35	182±31	149±25	143±34
5 til 10	72±20	44±9	52±10	47±6	60±9	65±13	65±8	63±24
Deschampsia, Oct. 2008								
Ohor	85±16	50±8	57±10	30±6	132±69	96±24	86±23	67±23
0 til 5	120±20	98±10	101±9	66±5	124±21	118±19	105±15	155±29
5 til 10	55±7	55±8	54±10	44±6	82±10	104±41	55±12	54±2

Ingrowth core new root production

The three in-growth cores were all of different ages: 6 months, 1 year and 1.5 years. The results are shown in figure 3 below. The significant P values ($P<0.05$) for the whole soil profile are shown in the graphs, and tendencies are also reported ($0.05<P<0.10$).
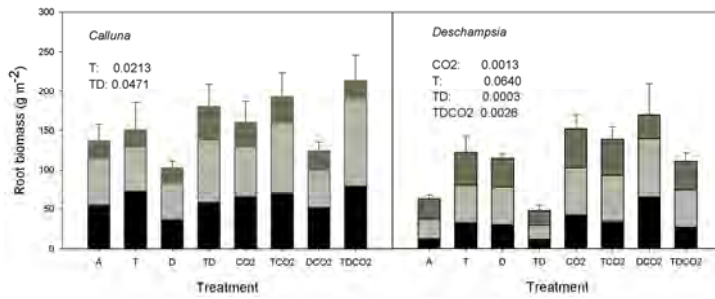
Root biomass from 6 months old in-growth cores (2007).
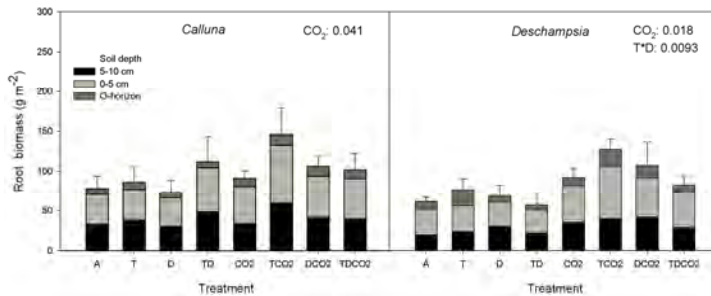
Root biomass from 1 year old in-growth cores (2008).
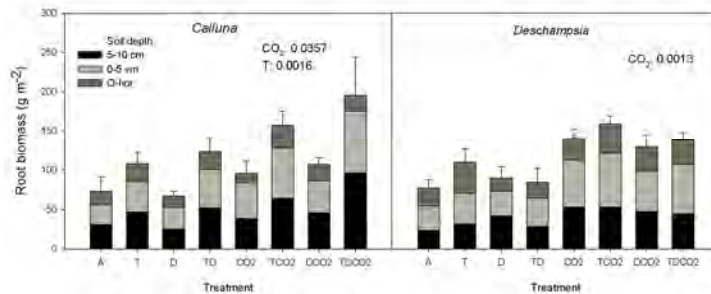
Root biomass from 1.5 years old in-growth cores (2009-2010).

Fig. 3. *Fine root biomass from three sets of in-growth cores.*

- The elevated CO_2 increased the root growth by 21% (non-significantly) and 65 % in *Calluna and Deschampsia* roots respectively, in the first set of in-growth cores.
- In the second in-growth cores, the positive CO_2 effect was 28 % in *Calluna* and 54 % in *Deschampsia*.
- In the last in-growth cores, the positive CO_2 effect was 50% and 58% in *Calluna and Deschampsia* roots, respectively.
- Warming generally increased root growth in *Calluna*
- TD generally decreased *Deschampsia* root growth

The elevated CO_2 effects or trends accounted for almost half of the effects seen in the in-growth cores, by increasing the root production. However 7 out of 26 effects were interactions (when separate soil depths were analyzed), which could not easily be interpreted from the single factor effects. In *Deschampsia* T×D and T×D×CO2 were the dominant interactions leading to treatment A and TD often being lower than the other treatments in the 6 months old in-growth cores.

Minirhizotrons

The results from the minirhizotrons are reported in Paper I, but root length and root production are shown in figure 4 and 5 below. The major findings from the whole period were:
- Warming decreased the root length in 8-15 cm soil depth.
- Elevated CO_2 tended to increase the root length in 15-50 cm soil depth.
- Drought decreased the root number, but CO_2 alleviated this effect.
- Warming increased fine root turnover in the last year of study.
- Elevated CO_2 increased the root production in spring 2009 (Fig. 5).
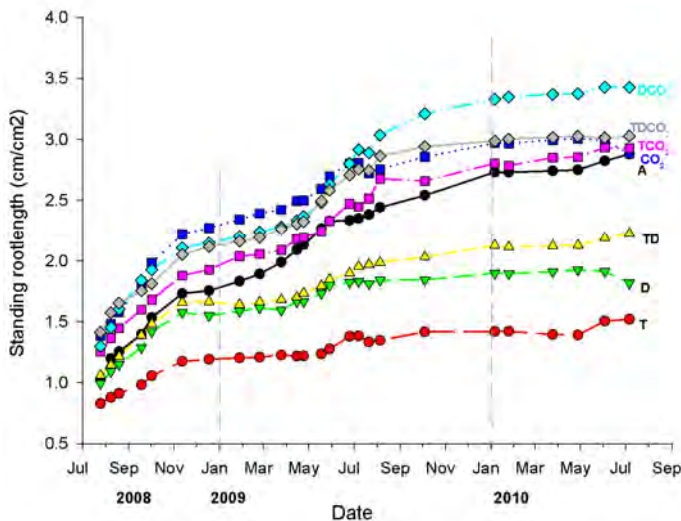
Fig. 4.
Standing root length observed in minirhizotrons (cm root cm^{-2} tube area) from August 2008 to August 2010 (8-50 cm soil depth). Error bars are omitted for figure clarity. Treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and the treatment combinations.
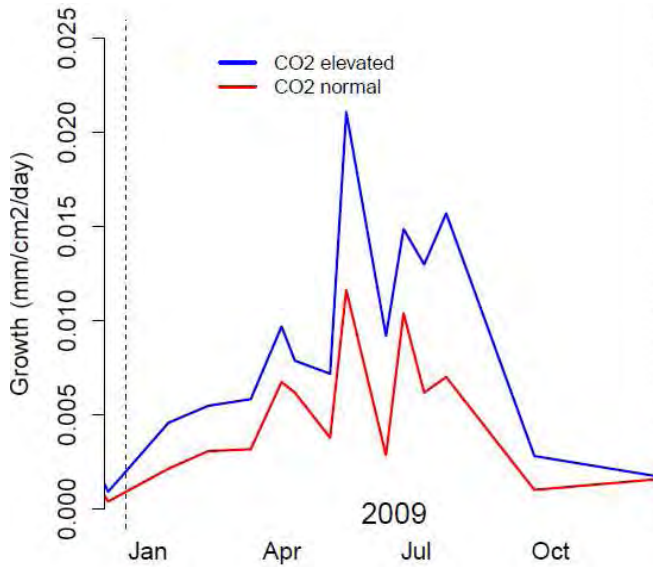
Fig 5. *Average production of root lengths is shown for plots with ambient and elevated CO_2. Production is measured as mm growth of root length per day divided by the area of the image. Though measures of increment were associated with a large sampling error, significantly larger increments were observed for elevated CO_2 plots at various samplings throughout the spring and summer of 2009.*

Nutrient uptake

High root ^{15}N or ^{32}P uptake in the root assays demonstrates N or P limitation in the plant. The results from the root assays are reported in paper II and III. The major findings were:

The winter assay in one year old in-growth cores:
- NH_4^+-N uptake was not affected by treatments in *Calluna* roots, and in *Deschampsia* in 5-10 cm depth there was a negative effect of drought, which was alleviated when in combination with elevated CO_2.
- NO_3^--N uptake was not affected by treatments in *Calluna* roots, but in *Deschampsia* the drought decreased the uptake, but CO_2 seemed to alleviate the drought effect.

The summer assay in 1.5 years old in-growth cores:
- The uptake of NH_4^+-N was not significant different between treatments in either soil depth in *Calluna*. In *Deschampsia* (5-10 cm) the uptake in treatments CO2 and DCO2 were higher than all other treatments, indicating that elevated CO_2 increased the uptake, while warming reduced the positive effect of elevated CO_2.
- P uptake in *Calluna* roots in 0-5 cm was significantly reduced in warming, and the uptake was significantly higher in both D and

28

CO2, compared to the combination with elevated temperature (TD and TCO2).

In *Deschampsia* roots, there was only a tendency for a positive drought effect in 0-5 cm depth, leading to higher P uptake.

Mycorrhiza

The results on mycorrhizal associations are reported in paper III, and the major findings were:

- Ericoid mycorrhiza did not show any clear treatment effects.
- The AM fungi colonization increased in elevated CO_2 in *Deschampsia*.
- The dark septate endophytes (DSE) in *Deschampsia* increased when drought and DCO2 were combined with warming in 0-5 cm, but in 5-10 cm DSE decreased in the drought treatment and drought in combination with temperature.
- The DSE in *Calluna* only responded in 5-10 cm depth with a decrease in drought.
- The fine endophytes of *Deschampsia* did not respond to any treatments in neither soil depth.

For all mycorrhizal associations, the colonisations were generally higher in 0-5 cm than in 5-10 cm for both plant species. This was similar to the root biomass, where the highest biomass was found in 0-5 cm for both species. However no clear correlation was found between root biomass and colonization, except for a significant positive correlation between percentage root length colonized and *Deschampsia* root biomass in 0-5 cm in (across all treatments, data not shown).

Root quality, N and P pools

The results on root P and N concentration and C:N ratio are reported in paper II and III. The major findings were:

- Elevated CO_2 decreased the root nitrogen concentration in both species, except in *Calluna* in the summer study in 2010.
- Drought increased the N concentration in *Calluna* in the summer time.
- C:N ratio increased under elevated CO_2 of both species, except in summer time where drought decreased C:N in *Calluna* roots.
- Root P concentration did not respond to the climate treatments in

neither species.
- In the summer assay the belowground plant N and P pools increased with elevated CO_2 in both species, and with warming in *Calluna*.

The root nitrogen concentration of both species across all treatments was lower in summer than in winter time. This point to a retranslocation of N at the end of the growing season from aboveground parts to belowground parts, as also seen in the same species reported by Aerts and Heil (1993). For both species, autumn senescence of leaves results in N being translocated to the root system for storage.

Root litter decomposition

Litterbags studies showed slow root decomposition at the experimental site, which suggest that many roots may have died long before they disappear. After almost 3 years the mass loss of root litter in *Calluna* was only 39% and 45 % in *Deschampsia* in control plots.
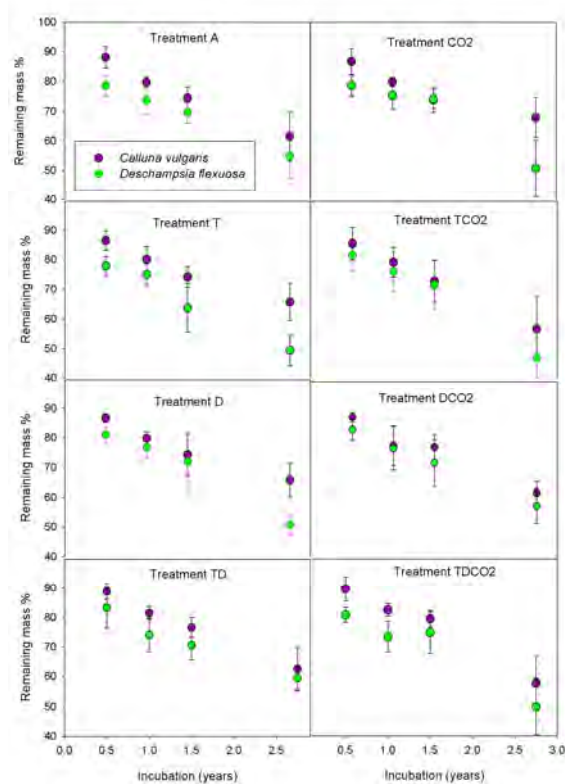
Fig. 6. Remaining mass (%) of Calluna and Deschampsia root litter incubated in soil in 6 months, 1 year, 1.5 years and 2.75 years (mean± std). The treatments are A (ambient), CO₂ (elevated CO₂), D (drought) and T (elevated temperature) and all treatment combinations.

Root litter from *Calluna* and *Deschampsia* was collected adjacent to the experimental sites and incubated in mesh-litterbags in every plot in the treatments, for a period of up to 2.75 years (t). The decomposition constant (k) was calculated based on % remaining mass of the litter (y) (Andresen *et al.* 2011a):

$$y = 100*e^{(-k*t)}$$

Root litter from *Calluna* had generally slower decomposition than *Deschampsia*, as also expected due to higher lignin content in *Calluna* roots.

Table 3. *Decomposition constant after 1 year and after almost 3 years of litter incubation for Calluna and Deschampsia.*

TREATM	A	T	D	TD	CO2	TCO2	DCO2	TDCO2
Calluna								
K-value 1 yr	0.227	0.222	0.225	0.205	0.225	0.234	0.247	0.193
K-value 3 yr	0.177	0.152	0.153	0.171	0.141	0.206	0.172	0.201
Deschampsia								
K-value 1 yr	0.304	0.288	0.265	0.300	0.283	0.275	0.258	0.310
K-value 3 yr	0.217	0.257	0.247	0.189	0.247	0.275	0.198	0.254

The lower K value after 2.75 years compared to the first year reflects the faster decomposition in the earlier phases, as reported by Harmon *et al.* (2009). For *Deschampsia* drought had a negative effect on decomposition after 2.75 years of incubation (P=0.0301). Warming and elevated CO_2 in combination significantly increased the decomposition for both *Calluna* and *Deschampsia* (T×CO2, P=0.0445 and P=0.006, respectively).
The combination of warming and drought tended to decrease the decomposition in *Deschampsia* root litter, indicating the influence of the prevalent moisture conditions to the decomposition.

SUMMARY DISCUSSION

This study investigated the root responses to a future climate with regards to root growth and distribution, nutrient uptake, mycorrhizal colonization and root turnover. The use of the different methods used in this study and their treatment responses are first discussed below. The research questions asked in the introduction are further addressed, and used to synthesize the root responses to climate change, with the contributions from paper I, II and III, and some of the data presented in the previous results paragraph.

The major purpose of this thesis was to study the root dynamics belowground in a future climate by using methods, which minimized the disturbance of the site. Root studies are difficult, and when the amounts of destructive root sampling are limited, the studies get even harder.

To be able to upscale minirhizotron images to root biomass, specific root length (SRL) is needed. Root diameter in forest changed during the study with elevated CO_2 (Pritchard *et al.* 2008), and highlights the importance of taking soil samples during the minirhizotron image acquisition, to get SRL and to be able to estimate biomass. This was not done in our study and we are therefore not able to convert root length to biomass. However, the responses to the treatments from the different methods can roughly be compared to see if they are in accordance (see Table 4 below).

When looking at the biomass from July 2007, (two years after treatments began), this represents the initial biomass before minirhizotron tubes were installed. At that time, the CO_2 response in 30-70 cm was more than 50%, but not significant. Three years after, the response in root length in the minirhizotrons in 25-50 cm was also more than 50% and significant at the end of the study. This suggests that the minirhizotrons resembles the biomass response, although root length is not converted to biomass. This is assuring, as the minirhizotrons are not changing the response to treatments. The depth distribution could have been measured by sequential soil coring in all consecutive years, if space was not limited. However the advantage of the minirhizotrons, besides being non-destructive, is the ability to study the dynamics of the root system, i.e. production, root lifespan and death. Longer duration of the minirhizotron image acquisition in this study would enable us to get estimates of root lifespan. As the minirhizotron data suggest, the treatment responses and the significance got stronger with time of study.

Summary table 4. Mean biomass (g m⁻²) from soil coring and production estimates (g m⁻²) from in-growth cores and minirhizotron initial standing crop before minirhizotron installation, root length (mm/tube image area) data shown as response ratios. The response ratio is defined as: (elevated-ambient)/ambient × 100, where 'elevated' represents elevated CO_2, elevated temperature or drought treatments (in all 4 combinations). The biomass values are only for the single control treatment 'A', used as a reference, when biomass values were available. Significant effects are marked in bold (P<0.05), tendencies with '†' (0.05<P<0.10).

Species, soil depth (cm)	Sampling date	Treatment A biomass (g m⁻²)	Warming response %	Drought response %	Elevated CO_2 response %
soil cores (g m⁻²) O-hor -10cm					
Calluna	April 07	423±86	+ 20	- 7	- 5
	Oct. 08	334±59	- 15	- 3	+ 2
Deschampsia	April 07	300±64	+ 7	- 15	+ 20
	Oct. 08	259±37	- 10	**- 23**	+ 30†
Ingrowth cores (g m⁻²) O-hor - 10cm					
Calluna	1/2 yr, 2007	137±21	**+ 41**	-3	+ 21
	1 yr, 2008	78±16	+ 29	-3	**+ 28**
	1.5 yr, 2010	73±18	**+ 70**	+ 14	**+ 50**
Deschampsia	1/2 yr, 2007	62±5	- 16 †	- 7	**+ 65**
	1 yr, 2008	62±5	+ 4	- 12	**+ 54**
	1.5 yr, 2010	77±11	+ 13	- 9	**+ 57**
Initial stand. crop (g m⁻²)					
Ohor-10 cm	July 07	428	- 12	- 9	+ 13
10-30	July 07	122 ±28	- 5	+ 4	- 1
30-70	July 07	46±17	- 26	+ 9	+ 57
Minirhizotron mm length/tube					
8-15 cm	July 2010		**-53**	- 2	**+ 2**
15-25 cm	July 2010		- 10	+ 1	**+ 36**
25-50 cm	July 2010		+ 15	+ 10	**+ 51**

The results from the in-growth cores show a stronger response to the treatments in general compared to the soil cores. The reason for the stronger response is probably due to the soil in-growth cores being root free from the start. This gives an opportunity to increase the root production, and especially in elevated CO_2 where there is extra C allocated to the root systems, the root biomass can increase. Besides that, the standing biomass might have a lot of older, persistent roots (as also observed in minirhizotrons) that 'dilutes' the treatment responses of new growth, compared to the in-growth cores. A lower standing root biomass from in-growth cores compared to soil cores was also observed by Neill (1992).

For the in-growth cores the large inter-annual variation made the responses different from year to year, except for the CO_2 effect which was

consistently strong. The higher root production in 2007 corresponds well with observations of the aboveground *Calluna* biomass, which also increased more in 2007, than in the other years of study (Kongstad *et al* 2011), due to higher precipitation (Table 1).

Both the root litter bags and the observed persistence of roots from minirhizotrons suggest that lifespan are long, and decomposition is very low. Minirhizotrons observations of root decomposition were found to be greater than that observed by buried bag studies (Hendrick and Pregitzer, 1996), but in our study there seems to be a good correspondence between the two methods.

Root biomass and length response to CO_2, warming and drought

The root length measured by minirhizotrons increased steadily over the two study years, indicating that the root system was not in a steady state (Paper I). Some explanation could be that the aboveground standing biomass also increased from 2008-2010 across all treatments (Kongstad *et al.* 2011). The increase was due to increase in *Calluna* biomass reflecting the natural succession in managed heath lands.

The measured root length indicated a strong response to future climate, with elevated CO_2 having the strongest effect on root length, whether alone or in combination with warming or drought. The minirhizotrons did not measure the upper ca 8 cm of the soil, but as the in growth core data suggest, the CO_2 effects was also apparent in the upper part of the soil.

The warming treatment decreased the root length in the upper part of the soil (8-15 cm), while the in-growth cores in *Calluna* most often increased the root growth in the upper 10 cm of the soil profile. Although minirhizotrons did not measure the upper 8 cm, the contradictory results are interesting. As *Calluna* responded most to warming, the differences seen in minirhizotrons might be due to differences in root distributions between *Calluna* and *Deschampsia*. Although it was not possible to identify species, I speculate that *Calluna* roots might be growing in the upper part of the soil, while *Deschampsia* might be more responsible for the deeper roots. This could explain the differences in the temperature treatments between in-growth cores (warming having a positive effect of *Calluna* roots) and minirhizotrons (having a negative effect on 'mixed' roots). The fine roots of *Deschampsia* might be growing deeper, to get access to more nutrients and water. The drought and warming treatment in combination (which is the treatment with the lowest SWC) was seen to have a negative effect on *Deschampsia* roots in the first in-growth cores, contrary to *Calluna* roots which was not negatively affected, and hence might not be limited by water to the same extent as the

grass. It would also make sense that the two dominant species are not competing for water and nutrients in the exact same soil layers.

Interestingly, except for reproductive structures no persisting changes in aboveground plant biomass were observed in response to elevated CO_2 (Kongstad et al. 2011), and most of the extra carbon assimilated (Albert et al. 2011a; Albert et al. 2011b) seems to be allocated belowground as a response to elevated CO_2 in this heathland. This indicates that the ecosystem might be in a transition period, where the greater exploitation of deeper soil layers is a response to limited nutrients. The rooting space in the upper part of the soil is probably 'occupied', and hence the only way of getting access to nutrients is by growing deeper. Deeper rooting distributions could minimize the expected increased nutrient leaching in the future found by Larsen et al (2011). The deeper root growth may result in changes over time in the ecosystem carbon storage, aboveground biomass and species composition as well. If the deeper rooting distribution is due to dominance of *Deschampsia* roots, this suggests that these ecosystems will be different under future climate conditions.

The drought did not have as much effect as expected, and only the root number was negatively affected by drought. A drought effect was also observed at the site on the enchytraeids biomass two years after the treatments began. Enchytraeids (white earthworms) contribute to the decomposition processes and nutrient mineralization and may control the quantity of microbial biomass in soil (Andresen et al. 2011b). Under elevated CO_2, increased C allocation belowground will typically stimulate the activity and growth of soil microorganisms and provide increased food available for enchytraeids (Maraldo et al. 2010). Increased C availability is often reflected at higher trophic level in the soil food web as the microorganisms are grazed down by soil mesofauna (Schmidt et al. 2002; Ruess et al. 1999). Drought was the main limiting factor on the enchytraeid biomass in this heathland, but this was alleviated by elevated CO_2 (Maraldo et al. 2010). This is similar to the negative drought effect observed on the root number, which was also alleviated by elevated CO_2. This also shows that the treatment effects are consistent across several ecosystem processes.

Root nutrient uptake response to elevated CO_2, warming and drought

Beside soil nutrient status the supply to aboveground plant parts are dependent on the root length available for nutrient uptake in relation to plant demands, as root length is an indicator of nutrient absorbing capacity (Mackie-Dawson and Atkinson, 1991).

As the results from the minirhizotrons showed, the root length increased

in response to elevated CO_2, supporting the data from increased root production in the in-growth cores, assuming the higher biomass also equals higher root length.

The root nutrient uptake was studied in root in-growth cores retrieved both summer and winter time with NO_3^--N, NH_4^+-N and PO_4^{3-} for *Calluna* and *Deschampsia* roots in different soil depths (Paper II and III). For the winter uptake study of NH_4^+ and NO_3^- uptake, there was a difference in the size of uptake between the two depths, where the highest uptake in both species was observed in 0-5 cm depth. The larger uptake may reflect a higher nutrient availability in the top soil layer due to recently produced litter of above ground plant material and an assumed on-going microbial activity due to sufficient water supply and no frost. These findings support that uptake of nutrients during winter do occur in this kind of ecosystem as found by Andresen and Michelsen (2005).

An earlier study at the site by Andresen *et al.* (2011b) indicated that the surface roots of *Calluna* might be more active in symbiotic N uptake than deeper roots. However, although the mycorrhiza colonization decreased with soil depth, the size of the NH_4^+-N uptake per unit root was similar between soil depths in the summer assay.

The summer root nutrient uptake of N and P indicated a weak positive CO_2 effect of NH4 in *Deschampsia* roots (indicating high N demand), and a lower P uptake in *Calluna* in response to warming (indicating low P demand) (Paper III). This N response and the higher N and P pool in *Deschampsia* corresponds to an earlier study at the site by Andresen *et al* (2009), who showed increased nitrogen acquisition (^{15}N labelled glycine *in situ* study) by *Deschampsia* in response to the single treatments of elevated CO_2 and warming. Andresen *et al.* (2010) found increased growth of *Calluna* plants in response to drought, followed by larger N uptake as shown by enhanced recovery of ^{15}N. They suggested that drought is beneficial for *Calluna* by inhibiting the competition from microbes. This is in agreement with our study where we observed higher root N and P concentration in *Calluna* roots in response to drought in 2010. Andresen *et al* (2010a) suggest that *Calluna* will benefit more from a warmer climate, as nutrient limitation of *Calluna* was counteracted by warming, as also seen by the low P demand in our study.

The soil samples from our study were taken 3 weeks after the drought period ended, and the tendency for the higher nutrient concentrations in *Calluna* could be due to rewetting and microbial lysis and nutrient release, which would result in a lower competition for available N and P between plants and microbes (van Meeteren *et al.* 2008). Drying-rewetting of soils increased the amount of water soluble phosphorus, predominantly in the organic form as it was released from microbes (Turner and Haygarth, 2001).

As highlighted by Cavagnaro *et al.* (2011), there is a need for further in-

vestigations of AM role in improving the plant N acquisition under elevated CO_2, and we included mycorrhizal colonization of both plant species in the summer uptake studies of N and P.

AM roots are often more efficient in nutrient acquisition per unit root length compared to non-mycorrhizal roots, as the fungal mycelium can absorb nutrients beyond the zone depleted by the root uptake, and exploits a larger soil volume than the plant root alone (Smith and Read, 2008).

It has been hypothesized that the relative cost to the plant of forming mycorrhizal associations are reduced under elevated CO_2 (Treseder, 2004), and we therefore expected higher mycorrhizal colonization in CO_2 treatments. Also higher colonization in response to warming and drought was expected. We found higher AM colonization in elevated CO_2 as expected but not warming and drought. However the hypotheses were based on a very small number of studies and not many field studies have investigated the effects of warming and drought on mycorrhiza.

The belowground *Deschampsia* N and P pool increased in response to elevated CO_2, while the N concentration of the roots decreased. This indicates that *Deschampsia* might be more N limited than *Calluna*, and maybe also more P limited as *Deschampsia* had a higher P demand than *Calluna*, which could explain the higher 'need for' AM colonization. The higher arbuscular mycorrhizal colonization might be a plant strategy for increasing the P uptake, and might also explain why we did not find decreased P concentration despite high root growth in CO_2 treatments. An increase in mycorrhizal association under elevated CO_2 would result in P tissue concentrations being little affected by elevated CO_2 in AM plants (Gifford *et al.* 2000), also observed by Conroy *et al.* (1990).

The ErM did not respond to the treatments as we expected, except for a decrease in the single warming treatment. In nutrient limited ecosystems, the elevated CO_2 and temperature treatment would be expected to increase ErM. However, when considering our data from the summer 2010, *Calluna* do not seem to be very nutrient limited in the warming or CO_2 treatment, supported by the findings of higher root N pool, lack of increased NH_4^+-N uptake and lack of N dilution in roots in response to warming and CO_2. Hence the ErM colonization might be sufficient for the requirements of nutrients at this time. However, to sustain the observed root growth and increasing nutrient pools in a future climate, higher concentrations of plant available nutrients are needed. To get an idea of the extra N needed, a rough calculation was made from the responses in the summary table 5. Using the 'mixed vegetation' biomass from the ambient treatment in July 2007 as a starting point, the biomass would by July 2010 increase by app. 40% for the whole soil profile. This calculation was done by using the mean of the minirhizotrons response ratios across the whole soil profile. The biomass increase would result in an increase of 2.20 g m^{-2} in the N pool belowground during 1.5 years. The at-

mospheric N bulk deposition at the site was 1.35 ± 0.04 g N m^{-2} y^{-1} in 2007 (Larsen *et al.* 2011). This means, that the nitrogen deposition would probably not support all the extra root growth in response to elevated CO_2, unless the N deposition increases. Hence progressive nitrogen limitation will probably minimize the high root growth responses to elevated CO_2, unless the increased N demand can be met by additional supply. The observed increase in root length in elevated CO_2 might be a strategy for the plants to cope with increased nutrient demand leading to a long term increased N uptake on a whole plant basis, despite unchanged N uptake per unit root mass. The deeper rooting distribution as observed in minirhizotrons (paper I), suggests that the roots are mining for nutrients in deeper soils under elevated CO_2 (Iversen, 2010). Deeper rooting distributions have been observed in forests (Iversen, 2010; Iversen *et al.* 2008; Finzi *et al.* 2007; Johnson *et al.* 2006; Pritchard *et al.* 2008) but to my knowledge no such pattern has been observed in heathlands or grasslands before.

Root quality response

In both studies with in-growth cores, the root N concentration decreased under elevated CO_2 which resulted in an increased root C:N ratio in *Deschampsia*, while in *Calluna* the effect was significant only in winter time. This increase in plant C:N ratio has been observed in many plants with an average of 15% (Gifford *et al.* 2000). There has been studies in our experimental site, showing increased C:N ratios in leaves of both species (Albert *et al*. 2011a, 2011b) in response to elevated CO_2. Together with the root response this indicates that increased C:N ratio might be a whole plant response. The reduced fine root N concentration is most likely due to an increased photosynthetic assimilation of C, but also a decrease in the specific uptake rates of N by roots grown under elevated CO_2 could be an explanation. This appears to be caused by a decreased shoot N demand and a decreased ability of the roots to supply N (Taub and Wang, 2008). The higher C:N ratio of plant litter may lower the litter quality, with consequences for the decomposers. However, as shown by Cornelissen *et al.* (2007) leaf litter decomposition will be driven more by climate and concomitant shift in plant growth form composition, than changes of litter quality within the species. This probably goes for root litter as well, and a change in dominance between *Calluna* and *Deschampsia* will be more important than the observed differences in root litter C/N ratio in response to the climate treatments. However the conclusions by Cornelissen *et al.* (2007) were based on warming treatment and not CO_2 as where we observed major changes.

Root lifespan and root turnover in response to climate change

Changes in production and turnover of roots in response to elevated CO_2, temperature and altered precipitation could be a key link between plant responses and longer term changes in soil organic matter and ecosystem C balance (Norby and Jackson, 2000).

The estimated root turnover rate across treatments of 0.04-0.07 suggest that less than 10% of the root biomass is renewed every year, but as discussed in Paper I, this is probably an underestimate. However the litter bag study showed that the average remaining mass of all treatments was ca. 37% and 47 % after 2.75 years for *Deschampsia* and *Calluna* roots, respectively, which corresponds to a decomposition rate of 16% and 12 % per year. Both methods indicate that root turnover is slow in this ecosystem, which is important information as root turnover rates determine the C input into the soils (Handa *et al.* 2008) and is a central component of ecosystem carbon cycling (Gill and Jackson, 2000; Meier *et al.* 2008). Turnover rates of 0.64 and 0.96 for *Calluna* and *Deschampsia* roots, respectively, are reported by Aerts (1993), which is almost 10 times higher than our estimate. Evidence from recent studies using isotope labelling suggests that mean fine root lifespan may be at least several years (Matamala *et al.* 2003; Trumbore and Gaudinski, 2003). This agrees better with the findings of this study, where direct observations of roots in minirhiztrons showed that most of the roots lived or persisted more than two years. Root decomposition represents a significant C flux in terrestrial ecosystems, and both the minirhizotron data and litterbags suggest that roots are persistent to decomposition for several years, at least. This could result in more C being stored in the soil in the root biomass.

We are not able to answer whether root lifespan are changed in a future climate, but there is no indication of higher turnover in a future climate with elevated CO_2, as the observed positive effect of warming seems to be lessened by elevated CO_2.

Carbon balance

Terrestrial ecosystems play an important role in regulating carbon feedback to the atmosphere. IPCC (2007) projects that carbon removal by terrestrial ecosystems is likely to peak before mid-century and then weaken or reverse, which would amplify climate change. There is therefore a great need for experimental studies to feed models and inform assessments of how ecosystem carbon storage will change in the future. In particular, our knowledge of belowground processes are limited, and studies of root dynamics are needed

to increase our understanding of whether ecosystems will act as carbon sinks or sources in future climatic conditions.

Higher soil respiration was observed between 2005 and 2008 at the CLIMAITE site under elevated CO_2 (Selsted *et al.* 2012), which corresponds to the observed increases in root biomass. Soil respiration is linked to root biomass, turnover and litter production, and hence elevated CO_2 results in higher soil respirations (Pendall *et al.* 2004; Zak *et al.* 2000). However, the data on C fluxes report a net loss of soil C in the ecosystem, and Selsted *et al* (2012) suggested this higher soil respiration was a plant mediated feedback, i.e. increased root exudation and respiration combined with increased microbial rhizosphere activity. They also implied that changes in microbial community and hence accelerated soil organic matter decomposition could explain the responses to elevated CO_2. More rapid rates of soil respiration under elevated CO_2 signal greater C flux belowground, suggesting that greater plant growth provides organic substrates for microbial metabolism (Zak *et al.* 2000). Much of the extra C under elevated CO_2 seems to be allocated belowground, and part of that could be as increased root exudation stimulating the microbial community. The increased root length may overall result in more exudates in the CO_2 treatment. Results from Hu *et al* (2005) indicated that elevated CO_2 increased plant N acquisition, and most N originated from the non-extractable pools because of enhanced microbial activity. Further studies might help to conclude what the status of the C balance in this ecosystem will be like in the future.

Implications for this heathland in the future

Using the data we have collected in this study, the future for this heathland indicate an increasing belowground N and P pool, higher root growth, hence higher root biomass, and more roots deeper in the soil. *Calluna* seems not to be as N limited as *Deschampsia* – hence the threats against a grass dominated heath may come from other disturbances than the direct climate change anno 2075. This is speculation, but is supported by the aboveground resilience to the climate treatments, as observed by Kongstad *et al*. (2011). The ecosystem may be nutrient limited to an extent where *Calluna* performs better than *Deschampsia* at this point, probably because of its ErM. However, if N deposition, frost events or heather beetle attack increases in the future, the concomitant damage to *Calluna* might be advantageous to *Deschampsia*, and the species composition might shift. However, changes in rooting distribution might suggest that changes already might occur below ground, and this may lead to changes in aboveground species composition.

CONCLUSIONS

The results of the CLIMAITE project indicates that the short term responses to climate changes mainly takes place underground, and if not taken the belowground effects and root dynamics into account, the ecosystem might seem more resilient than what is actually the case. The findings from this PhD study are significant effects on root dynamics, belowground nutrient pools and the C balance in response to climate change. The research questions asked in the beginning are answered below.

How does root biomass change in response to elevated CO_2, warming and drought?
Does the vertical distribution of roots change?

- Elevated CO_2 increased the root length, production, the biomass and number of roots growing deeper in the soil. Increased amount of roots in the deepest soil layers can have possible consequences for soil carbon balance.
- The warming treatment decreased the root length in minirhizotrons in the upper part of the soil, possibly as a result of lower soil water content, higher mineralization and higher turnover.

Does root nutrient uptake match an expected increased growth, under elevated CO_2?

- The roots in the winter assay did not fully compensate the higher growth by a similar increase in nutrient uptake, as the roots were unsuccessful in translating additional carbon uptake into increased nitrogen uptake per unit root.
- Different treatments response between the two species were seen in the assay from the summer time; higher NH_4^+-N uptake in *Deschampsia* roots in elevated CO_2 (indicating high N demand) and a lower P uptake in *Calluna* in response to warming (indicating low P demand). The root N concentration of the grass, *Deschampsia*, was lower in elevated CO_2, although root growth and the uptake of NH_4^+-N per unit root increased indicating that the nutrient uptake does not fully increase in concert with elevated CO_2. Root P concentration was unlike the N concentration not affected by the treatments in any of the species.

- The associated fungal colonizers responded differently to the treatments. Elevated CO_2 increased arbuscular mycorrhizal colonization, while the ericoid mycorrhiza did not respond and the DSE of both species was negatively affected by drought.

Are there changes in root lifespan and root turnover in response to climate change? Is root litter decomposition changed in a future climate?

- We are not able to answer whether root lifespan are changed in a future climate, but the turnover estimate indicated an increase in turnover ($year^{-1}$) in response to warming. There is no indication of higher turnover in a future climate with elevated CO_2, as the observed positive effect of warming seems to be lessened by elevated CO_2.
- Root litter decomposition was very slow for both species. In *Deschampsia* root litter the drought had a negative effect on decomposition after 2.75 years of incubation, while for both species warming and elevated CO_2 in combination significantly increased the decomposition.

Further we conclude
- The results from summer 2010 indicate increased belowground plant N and P pools in response to warming and elevated CO_2. This could result in changes in plant competitive interactions as well as belowground nutrient pools in response to future climate change.

- Significant second and third order interactions were plenty, showing that main factor effects were often not additive, and that changes to multiple environmental changes cannot be predicted from single factor responses alone.

Future research perspectives

Some research topics were identified that could further help our understanding of root dynamics in response to climate change.

Future research should address how separation of roots by species in mixed communities might be undertaken, as this could help understanding the root community as a whole. One approach might be to use root boxes placed specifically under *Deschampsia* and *Calluna*, and *in situ* follow the root growth and the changes in appearance. Root order could also be followed and N content of several diameter classes could be determined and used for up scaling.

Root lifespan analysis, directly measured by minirhizotron was not possible because insufficient time had elapsed for a survival analysis to be carried out. It would therefore be of great value to continue the analyses using the minirhizotrons already *in situ*, together with other methodological approaches such as radiocarbon analyses. This would enable a better understanding of root dynamics to be obtained.

Another issue would be to get the Specific Root Length to upscale minirhizotron data to biomass – this can be done by scanning roots to obtain length and weight from destructive soil coring. More research is also required of mycorrhizal fungi responses and their role in improving plant nutrient acquisition in response to global change factors.

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APPENDIX 1

FINE ROOT SCANNING AND IMAGE PREPARATION USING MINIRHIZOTRONS AND SPECIALIZED SOFTWARE

INTRODUCTION

Belowground studies have long been delayed by the difficulty of measuring root systems (Hendrick and Pregitzer, 1996). One method to study root dynamics is by using minirhizotrons, where roots can be studied non-destructively and repeatedly in time (Gregory, 2006). This is done by remote sensing fine root growth using photographic image or scanning techniques. Specialized scanner or camera capture images of fine roots from inside and out of transparent tubes placed in soil.

Minirhizotrons allow for measurements of root diameter, longevity and length, but not root nutrients concentration or dry weight. Generally there is no easy way of converting root length observations from minirhizotrons to bulk soil estimates of root length, and often estimations from minirhizotrons rarely replicates the data obtained from soil coring (Gregory, 2006). Hence, to convert root length to biomass a conversion factor must be developed as well as some assumptions regarding depth of view (of the scanner). A depth of view of 2-3 mm has been used (Brown *et al.* 2009; Bernier and Robitaille, 2004) although this is an arbitrary number (Gregory, 2006).

Another method could be to measure root biomass and nutrient content with soil cores and then use the data of production and mortality from minirhizotrons to estimate belowground productivity and turnover (Hendrick and Pregitzer, 1996).

However, before getting any results, image acquisition and the subsequent image analysis have to be done first. The most known root acquisition devices on the market are the root camera from Bartz Technology Corporation (USA) and the root scanner from CID Inc. (USA). The camera has en image area of 1.35 cm x 1.8 cm, while the root scanner image is 21.6×19.6 cm. The larger scan image is valuable in many ecosystems, but it might also lead to problems in soils with high root density. In that case, it will be necessary to take out sub-samples to get a possibility to track roots individually in a realistic time frame. As the root scanner is rather new on the market compared to the root cameras, the increasing use of the root scanner might create problems similar to ours, by getting too many roots per image, hence prolonging root analysis indefinite.

This paper describes the practical processes around establishing studies of root growth using minirhizotrons in soils with many fine roots, and the development of specialized software to deal with the image preparation. Rec-

ommendations, observations and best practices based on experience from this Ph.D study and the use of the Contract Image Sensor (CIS) typed root scanner with model number CI-600 from CID. Inc., are described and discussed in the following chapters.

MATERIALS AND METHODS

Installation of tubes

Before installing any minirhizotron tubes in the test site, it is important to have some kind of mark on the tubes, as the tubes from CID Inc. come 'clean'. If a software tool should automatically recognize the image location in the tube, by doing automated image position referencing or image mosaicing, reference points are required. This can be done by making dots using permanent thick light coloured ink/paint. No software is capable of doing automated image positioning on un-dotted images, due to lack of contrast and colour difference in a soil/root image. Space between dots on tube should be approximately 10 cm, but the precision of dot location on tube are of minor importance.

The minirhizotron tube installation is a critical aspect (Hendrick and Pregitzer, 1996; Volkmar, 1993), as a good tube-soil contact is a prerequisite for getting realistic estimates of root growth and to create the best possible conditions for observing fine roots.

For this project, 1 m long minirhizotron tubes and soil corer from CID Inc. USA where used. The tubes delivered had smaller outside diameter compared to the soil corer outside diameter. It is very critical that the diameter of outside tube is approximately 1-2 mm smaller than the soil corer outside diameter, thereby reducing gaps between tube and soil. Large gaps will result in unclear images, moisture on the outside of tube, uneven root growth in gaps and build up of sand between tube and soil. Condensed water makes the images useless as scanner cannot focus on roots. Further more, CIS typed image scanning technology get the best results with a short distance from scanner head to surface. However, due to lack of contrast between this heathland soil and the roots, it turned out to be easy to observe the roots when they grew in the gap, although it does create an artificial environment.

The recommendation from this experiment is, if possible, to make a few test tube installations in different types of soil on location and test scans, before installing all tubes. However it is hard to know whether the soil will settle around the tubes with time, but it did not happen in this study. To make sure you get a good soil-tube contact on topside of tube, adding extra soil around top of tube might reduce soil gaps after coring and installation.

This could also reduce the risk of having insects hiding in the gap between tube and topsoil, and also reduce the risk of having water percolate alongside the outer tube surface.

The minirhizotrons were installed in an angle of 45 degrees to vertical. For non-woody species an angled tube installation is recommended to avoid roots growing preferentially along the tubes (Johnson *et al.* 2001). However, roots in this study seemed to concentrate at the downside of the tubes, probably due to a better soil-tube contact there or higher soil moisture.

The fine roots in the upper 10 cm of the soil are not accounted for in our experiment, as the upper images were discarded due to poor image quality, which might also bias the estimates. The images were poor due to roots mixed with dark brown organic material, making the contrast too small to separate from each other.

The tubes were left for one year before image acquisition started. Johnson *et al.* (2001) recommends a waiting period between minirhizotron tube installation and root data collection of 6-12 months (Johnson *et al.*2001), although other reports of longer waiting periods of up to 6 years (Aerts *et al.* 1992; Iversen *et al.* 2008; Milchunas *et al.* 2005). As reported in paper I, one year of calibration does not seem to be enough in slow growing, nutrient poor ecosystems like heath lands. This should be kept in mind in future studies.

Image acquisition and preparation

During image acquisition, the root scanner is inserted inside the minirhizotron tube at known depths and orientation, and connected to a laptop computer. The scanner head revolves 345 degrees and records the interface between the clear tube and the soil. The result is a 21.6 x 19.6 cm picture in colour pictures (300 dpi). At each tube, three sequential images were taken at depths approximately 5-19 cm, 20-34 cm and 35-48 cm vertical depth. As there were too many roots to track in each image, four to five minor sized cropped images, called tiles, needed to be cut out from each raw scanned image. The sizes of these were: 1.5 x 3 cm in the upper image (which was the most populated area) and 2 x 3 cm at the 2 lower images. This made a total of 15 small images along the depth profile, with 2.5 cm apart in the first and 1 cm apart in the 2 lower images.

Approximately 3660 scans ('raw' images) resulted in 17.860 images tiles to be digitized and analysed. The image preparation would take several man months to complete if done manually. Therefore it was necessary to design and develop specialized software to semi-automate the image preparation processes.

First programs were written to realign all pictures to make sure the sub-samples were cut out from the exact same spot in order to follow the same individual roots continuously over the measurement period. This was needed as the scanner was not positioned in the exact same spot in the tubes from one sampling to the next (+/- 1 to 10 mm of difference), and as we had no reference marks in the tubes.

Specialized software for image preparation and analysis

Three software tools were needed to process the 'raw images'. Each tool supports a separate part of the process.

- Images renaming tool (Image_rename), which is used to rename the field work names into names used by the root program Rootfly, used for the final analysis.

- Images referencing tool (Root align), before cutting out subsamples, the images must be aligned.

- Images cropping tool (Crop_images), which cut out subsamples from the raw image.

The Images referencing tool and the Images cropping tool use a Microsoft Access database developed for the purpose of handling the process related data and exchange information between the tools.

The process to complete the image preparation and analysis consists of the following steps.

Step 1: Rotate images
Step 2: Rename all images to a unified naming convention
Step 3: Create image management database
Step 4: Position reference images
Step 5: Crop images into manageable tiles

STEP 1: ROTATE IMAGES

The output from the root scanner is a horizontal image, which has to be rotated to resemble the soil profile and ease the tracking of roots and fit into the root program. It is therefore important that all images are rotated to a ver-

tical (portrait) position. This process can be automated using the freeware software IrfanView, by using the IrfanView tool 'Batch conversion' - 'Use advanced options'. Make sure the top of image is upright.

When doing field work, short, easy names are needed to speed up the scanning time in the field. However, when all images are acquired, image files should be renamed to a shared naming convention that is required by most root analysis tools. Usually images should be renamed according to the ICAP/Bartz naming convention, where the following should be included;

- Experiment name (whatever name you want)
- T for tube #
- L for location, ie. image # where 1 is the first upper picture taken
- Date and time of pictures taken (date separated by ".")
- The last number is the session number
- Finally the initials of the person who gathered the data
-

Files format used during scanning is bmp (bitmap image file).

The renamed filename should follow this naming convention:
Experiment_T#_Image#_Date_Time_session_intials.bmp

An example from the CLIMAITE site is:
climaite_T001_L001_25.08.2009_1248_001_mfa.bmp
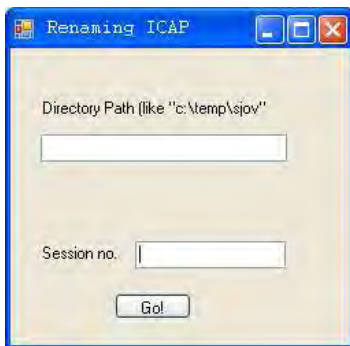
Efficient renaming has been done by specialized software developed specifically for this task (Renaming ICAP). The software structures filenames correctly by inserting date, time etc. in an automated process. Renaming is done per session. All images for a scan session should be located in one folder, and then renaming of several hundred files takes less than a second. The figure on the left handside is the specialized software developed for renaming images to the ICAP naming convention. All the renamed images are placed in one folder. The rename tool is developed in Microsoft Visual Studio using development language C#.

STEP 3: CREATE IMAGE MANAGEMENT DATABASE

A Microsoft Access database should be created wherein all processing information will be located with basic metadata on images. The database is used by the 'image referencing tool' and the 'Images cropping tool', described in step 4 and 5.

Using a database instead of text files or simple metadata containers, ensure easier changes in data structures, faster software customizations and less data and software errors.

Database is called "RootAlignAndCut".
It includes a set of tables and queries used by the tools. There are the following tables (see Fig. 2).

- 'Import_location' which contains the initial imported filenames of renamed image files.
- 'Scan_test' contains initial metadata about each image.
- 'Referencedscan_test' lists the result of each image reference. The x and y vector for which the image cropping should be projected.
- 'Only_loc2_2x3cm' table lists all the tubes and their cropping tiles. A cropping tile is defined by a startX, startY, stopX and stopY and a cropping name. This table needs to be complete for all tiles required for all tubes, before cropping can be executed.

- and following queries:

- 'Scan update from filename' extracts scanned files information from file name and inserts them into the table 'scan_test'.
- 'Join scan_&_referencedscan' is a join query used by the referencing tool to list the images that has been referenced. It is used by both the referencing tool and the chopping tool.
- 'NextTubeLocationLastSession' is a query that returns the next image to be referenced. Is used by the referencing tool.
- 'NotReferencedYet' lists the remaining images to be referenced.

To make a new database for referencing and chopping, complete the following steps.

- Put all files (up toup to several thousand) into one folder
- Make sure to include the reference pictures. These pictures will automatically be used as reference pictures based on their higher session number (see Step 4).
- Open command prompt cmd in windows. Type "cd" followed by path to image file directory. Type "dir > files.txt" and all filenames are piped into textfile.

- Using a text editor, clean up text file so only the filenames (ICAP) are listed by deleting header and footer.
- The file names (ICAP) are imported into the Access database table 'Import_location' using Access. Import only the column 'filename'.
- The original filename listed in 'import_location' table is split up into: field Experiment, Tube, Location; datetime, session and gatherer. This is done by running query 'Scan update from filename' from within Access.
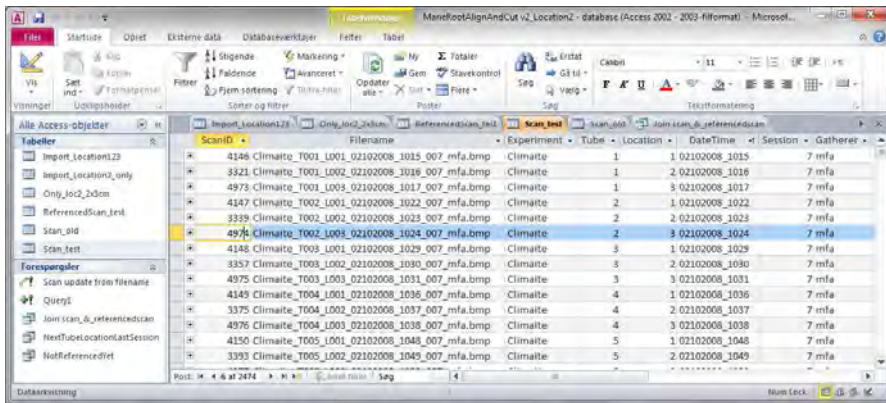
Fig.2. *The Access database created with different tables used. See above paragraph for explanation. The 'Scans_test' table showed contains initial metadata about each image, named according to the ICAP naming convention.*

STEP 4: POSITION REFERENCE IMAGES

To ensure all subsamples are cut from the exact same area on all images, there need to be a reference between all the images, in order to align the images. In minirhizotron tubes, which do not have any grid or other recognizable patterns, a manual reference is therefore needed before cutting out subsamples from the larger image. In this process, we used the images from the final session as a reference image – in that case we could be able to find a characteristic 'spot' in the image, which would be there throughout all sessions.

Make a reference point to align all images before cropping

The images referencing tool (Root align) is specifically developed for the task of referencing root images. The application requires a minimum 24" inch widescreen to make it possible to identify fine roots on images.
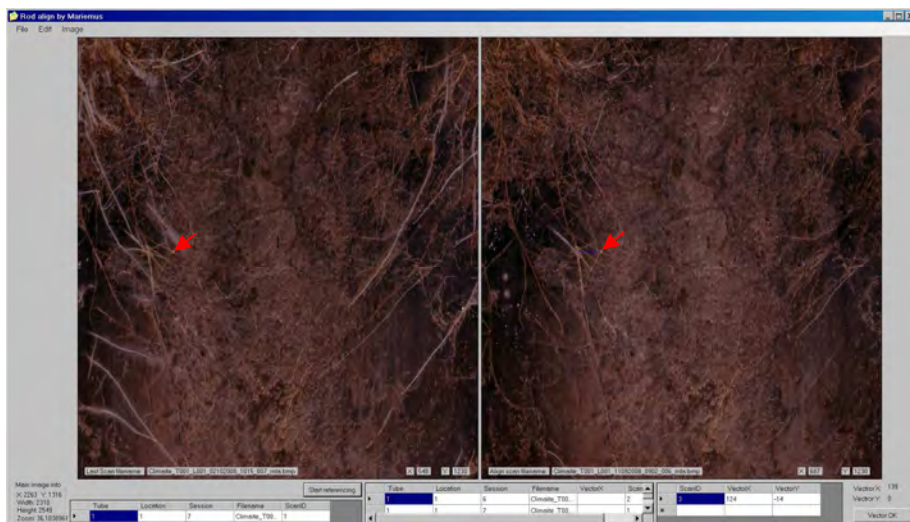
Fig. 3*. The 'Root align' specialized software developed, with a reference image at left and a 'to-be-referenced' image at the right. The reference point is placed in the cross section of two roots (see arrows), which were visible and placed in the exact same position throughout the whole measurement period.*

Process for using the 'Root align' tool:

- In current version of the tool, the connection to database and location of files are hardcoded into the software. Before using the tool a software developer should hardcode this information to fit the computer being used.
- When opening the application, press button 'start referencing', and the images: 'reference image' and 'the to be referenced image' are shown, together with some information tables below (see Fig 3)
- The reference tool runs through all available sessions for one tube location before moving to referencing the next location (i.e next soil depth or tube).
- On the image from last session, the reference image, you should find a reference point that is not changing through all sessions

and can easily be detected on all subsequent images (patterns in soil or roots that live through all sessions).

- On all images from the first to the last session the same reference point should be placed on all images as precisely as possible (do it manually with the mouse) by always comparing it to the reference session.
- The difference between the reference picture and the rest will be observed by the 'Root align'-program, which calculates the vector changes (how many pixels is the picture moved from the reference session). This means that when all pictures have the same reference point, the vectors can be used to align all pictures exactly on the same spot. The vectors will be saved in the database.
- Make sure all pictures seem to be aligned perfectly – if some vectors are more than a centimetre, the reference point might have been misplaced.
- Be aware that there is an overlap between scanned images of 0.5-1.2 cm on the top/bottom of the pictures.

The 'Root align' software is developed in Microsoft Visual Studio using software development language C#. An important component used for the development is the A.Forge opensource image processing component library (http://www.aforgenet.com/). AForge.Imaging is a library with image processing routines and filters.

STEP 5: CROP IMAGES INTO MANAGEABLE TILES

Cutting out images into manageable tiles (sub samples of images) might be necessary when using the CI-600 root scanner in ecosystems with many roots, as there can be several hundred of roots to track in each image.

- Decide the area you want to cut out of each original scanned image. The area should be no bigger than you'll preferably have 5-10 roots in each image, but this depends much on root density. Be sure to be able to zoom in on the roots –if it is too small you might have trouble seeing it clearly.
- The area should be similar on all images and depths – otherwise it will be biased due to the curvature of the tube if the area gets bigger. If there is a lot of difference in amounts of roots from deep to shallow layers, you might just increase the distance between the boxes instead of the area.

- The area of each tile (sub-sample) to be cropped is entered into the database table 'Only_loc2_2x3cm' from with Microsoft Access database. It is recommended to make an excel spreadsheet with vectors and the X and Y coordinates for start and stop for cropping. Then import this excel sheet into table 'Only_loc2_2x3cm'. Excel is faster for entering and copying coordinates for all tubes and locations. If scanned images do not have exactly same size, you can compensate by adjusting vector with same percentage difference in size. This could happen if scanner-settings are changed during field work, making the size of the image different from the rest.
- When root box area and distances have been entered into database, use the Images cropping tool to cut out the sub-samples, e.g. make a cut of 2x2 cm for every 2,5 cm all the way through the tube.
- New sub-samples are saved under the same ICAP names, although Location number starts from 100 instead of 1 (ie. L101 instead of L001), just to be able to separate them from the original version of the files.

In this project, images in upper part location, named L001, has 5 smaller images cropped in each big image, but the size is half of that in the other depths as there were many roots in the upper horizons (2 x 1,5 cm in L001 while L002 and L003 are cropped by 2 x 3 cm), see Fig. 4.
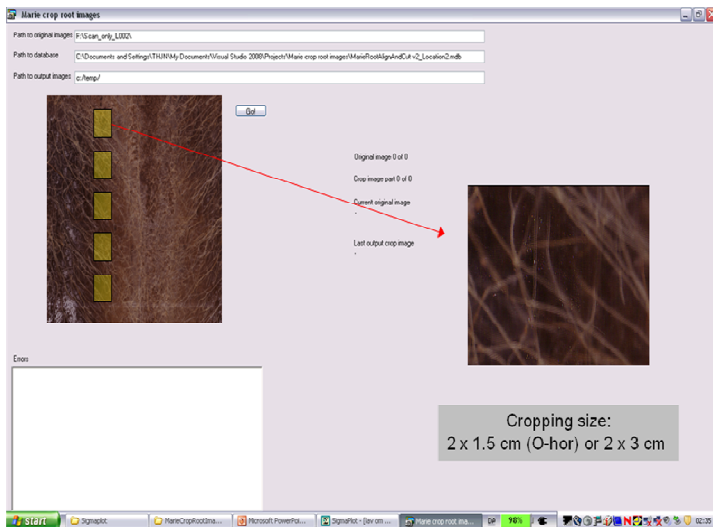

Fig.4. *Screen shot of the developed 'image cropping tool' showing the original image (as taken by the root scanner) and 5 'subsamples' that will be cut out.*

When running the 'image cropping tool', first enter the path to images, path to database and path to the output cropped tiles. Then press 'Go!' button. For cropping 1000 images into approximated 5.000 tiles the tool use around one hour. Any error in the cropping process, e.g. a crop area outside a referenced positioned image is listed in the 'Error' box.

The final part of the process is the root analysis, which is not part of this guideline. I used the free, open-source software application Rootfly, which is released under the GNU General Public License (GPL). (http://www.ces.clemson.edu/~stb/rootfly/).

DISCUSSION

There is a great bias between the easy image collection and the time needed to digitalize the images (Hendrick and Pregitzer, 1996), and in our case, to prepare the images for analysis. In ecosystems with many roots (as ours) a mini camera with a smaller image area might be more useful. However, in forest ecosystems with fewer roots, the root scanner would give valuable information as it is possible to follow the whole root system and verify the exact root order in a much larger picture. Furthermore, with a larger image you could avoid the difficulty of separating roots into different species (Hendrick and Pregitzer, 1996). To be able to distinguish roots according to species requires specific differences in root morphology, also at small scales. Roots that turn brown or develop a cork cambium make it difficult to distinguish roots from the soil background (Hendrick and Pregitzer, 1996), even for skilled operators of manual systems (Hendrick and Pregitzer, 1996). Due to the enlargement of our images, the image quality became poor and made it impossible to distinguish difference in roots between the species. To upscale into a biomass estimate requires that roots can be distinguished on the species level and the specific root length (SRL: m root g^{-1} root) for the dominant species are known.

There have been published papers with automated detection of roots (Zeng *et al.* 2006; Zeng *et al.* 2008; Zeng *et al.* 2010), but these are on bright young roots and with only few roots per image. The roots in our heathland were not bright, with little contrast and the image tiles often contained more than 15 roots, making automated detection impossible. Automated detection were investigated, but the tested image reconnection algorithms by A.Forge, need brighter more colourful roots, more image contrast and higher resolution in images to automate the detection of fine roots. Images filtering techniques using classic image processing methods were also investigated and tested. But the result showed that it was impossible to im-

prove image quality to ease automated and or manual root detection. A scanner scanning other light spectra than the visible light might be able to scan roots with a higher contrast, similar to terrestrial remote sensing satellites scans using infrared and ultraviolet spectra. This use of UV has been done by Smith and Zuin (1996) and Wang *et al.* (1995).

CONCLUSION

The main conclusion regarding root image analysis from this study is to be aware of the ecosystem going to be studied, before buying the minirhizotron equipment, and to be well prepared. If a high root density is expected, the smaller image size captured by a mini camera might be preferred, as there is a lot of work in taking subsamples out from a larger image, not to mention lack of image quality when scaling up. Taking out subsamples from a larger image is almost impossible without specialized software.

The tubes and soil corer should be tested together at the site to determine the best soil-tube contact. If possible, reference points should be marked on the tubes before installing them in the soil. This will ease the image referencing process, if sub sampling is needed.

A few test scans might also give an idea on sampling intervals needed to follow the root lifespan and death of individual roots.

The image analysis afterwards is very time consuming, but unfortunately there is no other possible and realistic ways of tracking roots (in soils with low contrast between soil and root), than visually, and manually analysing roots.

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PAPER I

CHANGES IN FINE ROOT GROWTH AND ROOTING PATTERN TO ELEVATED CO2, WARMING AND DROUGHT IN A MIXED HEATHLAND-GRASSLAND

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ABSTRACT

The responses of fine roots to climate change have mostly been studied in experiments manipulating single factors. Little attention has focussed on how such factors will interact and affect root system size and dynamics.
In this study, we investigated the combined effects of elevated CO_2, increased temperature and summer drought on root standing biomass and root length in a heathland. The treatments mimic the predicted climate conditions for Denmark 2075 and we report responses from the first five years after treatment application.

The most pronounced effect was caused by elevated CO_2, both alone and in combination with increased temperature and extended summer drought periods, and only few interactions were observed. Elevated CO_2 had a positive effect on total root length (+ 45%) due to more roots, leading to increased root growth in the future climate scenario.
The warming treatment decreased the root length in the upper part of the soil (8-15 cm), possibly as a result of lower soil water content, higher mineralization and higher turnover.

Elevated CO_2 increased root length in the deepest soil layers with possible consequences for soil carbon balance. As there is no major aboveground

plant community change at the site, our results suggest that ecosystem carbon allocation and storage will be different under future climatic conditions with more roots and particularly in deeper soil layers. This might have great implication for the C balance, dependent on whether a higher fraction of the extra root biomass will enter the recalcitrant pool of soil organic matter or whether root stimulated changes in turn-over rates of soil organic matter will counterbalance the input.

INTRODUCTION

Anthropogenic combustion of fossil fuels have increased the amount of CO_2 in the atmosphere, and the amount in 2005 (379 ppm) exceeds by far the natural range of the last 650,000 years, and the increase is expected to continue (IPCC, 2007). Further more eleven of the twelve years in the period 1995–2006 rank among the top 12 warmest years in the instrumental record and extreme events of both drought and heavy rainfall are projected (IPCC, 2007). These environmental factors are drivers of many important ecosystem processes, and changes in ecosystem function are therefore expected in response to changing climate. IPCC (2007) projects that carbon removal by terrestrial ecosystems is likely to peak before mid-century and then weaken or reverse. This would amplify climate change. There is therefore a great need for experimental studies to inform assessments of how ecosystem carbon (C) storage will change in the future. This requires understanding of above- and belowground pools and fluxes and their responses to rising CO_2 and a changing climate. Belowground studies have long been delayed by the difficulty of measuring carbon flux to the soil, which are important for modeling the C balance.

If an ecosystem should act as a C sink in the long run, the extra sequestered C need to be incorporated in a carbon pool with slow turnover, as soil organic matter (SOM) or wood (Adair *et al.* 2009). In forests, the large biomass aboveground would be the critical C sink (Gill *et al.* 2006), while in semi-natural ecosystems like grasslands and heath lands, soils would be the most important long term C sink. Heathlands account for 7 % of the European land cover (European Environmental Agency: http://www.eea.europa.eu) and in UK more than 100 Tg C is stored in the soil of dwarf shrub heaths (Ostle *et al.* 2009), making this an important ecosystem in the terrestrial carbon balance. Most of the net primary production in grasslands occurs belowground (Steinaker and Wilson, 2005), and root biomass often exceeds aboveground standing biomass by c. 4 times (Mokany *et al.* 2006). This means root litter contributes more to the SOM pool than the contribution of

shoot litter (Mokany *et al.* 2006; Arnone *et al.* 2000). Global fine root carbon equals more than 5% of all carbon contained in the atmosphere and assuming a conservative fine root turnover of one year, fine roots represent 33% of global annual net primary productivity (Jackson *et al.* 1997), hence root dynamics constitutes an important component of ecosystem C and nutrient cycling.

Fine root responses to CO_2

Many studies of fine root responses to elevated CO_2 have now been conducted in forests (Iversen *et al.* 2008; Iversen, 2010; Pritchard *et al.* 2008; Johnson *et al.* 2006), and grasslands or steppe (Adair *et al.* 2009; Anderson *et al.* 2010; Edwards *et al.* 2004; Higgins *et al.* 2002; Milchunas *et al.* 2005; Nelson *et al.* 2004) while studies of root dynamics in temperate heath lands exposed to elevated CO_2 are generally absent. Plant growth has often been shown to increase under elevated CO_2 (Rogers *et al.* 1994) with more carbon being allocated to the roots (VanVuuren *et al.* 1997; Fitter *et al.* 1997). However, negative responses of CO_2 are also reported. Lower root length in elevated CO_2 in a Californian grassland was reported (Higgins *et al.* 2002) and only 5 out of 12 grassland studies showed marked increases in root system size to elevated CO_2 (Arnone *et al.* 2000).

The positive effects of elevated CO_2 on plant growth in natural ecosystems can be limited if nutrient limitation constrain the carbon allocation to growth (Gill *et al.* 2006; Arnone *et al.* 2000). In the Progressive Nitrogen Limitation (PNL) hypothesis, available N becomes increasingly limiting as C and N are sequestered in long-lived plant biomass and soil organic matter (Luo *et al.* 2004). PNL is less likely to occur if there is sufficient N available in the system to match the increased C uptake and growth or if the extra N demand can be met by for example increased fine root growth and a deeper rooting distribution in soil stimulated by elevated CO_2, thus increasing root exploitation of deep N resources and prolonging the positive responses to elevated atm. CO_2 (Finzi *et al.* 2007; Jobbagy and Jackson, 2000). The observed increase in root production under elevated CO_2 is often greatest below 15 cm depth, resulting in a larger proportion of root biomass deeper in the soil, as shown in trees exposed to elevated CO_2 (Iversen, 2010; Finzi *et al.* 2007; Johnson *et al.* 2006; Pritchard *et al.* 2008; Iversen *et al.* 2008). The change in rooting depth in elevated CO_2 is mainly reported from forests (Iversen, 2010), while in short grass steppe and desert no effect of elevated CO_2 on deeper root distribution was observed (LeCain *et al.* 2006; Ferguson and Nowak, 2011).

Fine root responses to elevated temperature and drought

Increasing soil temperature stimulates root growth, when all other factors remain equal (Pregitzer et al. 2000). Warming often result in the environmental temperature being closer to the optimal temperature and might increase photosynthesis (Sage and Kubien, 2007), resulting in extra fixed carbon to be allocated belowground to sustain new root growth (Pregitzer and King, 2005). Increasing root biomass and length under rising temperatures allow the plants to exploit a greater volume of soil (Pregitzer and King, 2005), similar to the expected CO_2 response.

In a metanalysis of 85 studies, warming enhanced the aboveground biomass but did not significantly affect the belowground biomass (Wu et al. 2011). The non-significant belowground biomass response to temperature could be due to concurrent increases in root turnover, leading to more rapid cycling and an identical or lower standing biomass belowground. Root turnover has been reported to increase exponentially with mean annual temperature for fine roots of grasslands (Gill and Jackson, 2000). Due to higher root mortality, root growth is unlikely to be directly affected by increased soil temperatures as a result of global warming, at least in temperate areas (Edwards et al. 2004).

Drought conditions often occur when soil temperatures increases, due to increased evapotranspiration. Several studies show that drought decreases the rate of root length extension and lower the conductance (reviewed by Pregitzer et al, 2000). Decreased precipitation suppressed aboveground biomass, but there is insufficient data available to assess effects on belowground biomass (Wu et al. 2011). However, when high soil temperatures are associated with drought conditions, there is no reason to expect an increase in root growth (Pregitzer et al. 2000).

Responses to multiple factors

General responses to changes in temperature and precipitation and their combined effects are still not well understood. The interactive effects of warming and altered precipitation tended to be lower than the expected from the single-factor responses (Wu et al. 2011).

The increased WUE under elevated CO_2 and the increased evatransporation under elevated temperature might offset each other and together have an intermediate effect on root dynamics (Johnson et al. 2006). Water limitation is often shown to amplify the percentage response of plant growth to elevated CO_2, caused by the reduced transpiration under elevated CO_2 leading to improved soil–water status (McMurtrie et al. 2008). To determine the im-

portance of temperature-precipitation interactions on the C balance in the future, new experiments with combined temperature and precipitation manipulations are needed (Wu *et al.* 2011).

Although there have been many studies of root dynamics and response to CO_2, there are few studies of the effects of interactions among CO_2, temperature and soil moisture, but see (Garten *et al.* 2009; Shaw *et al.* 2002). The largest emphasis has been on elevated CO_2 alone, due to the presumed direct feedback between root turnover responses to elevated CO_2 and the cycling of CO_2 through ecosystems and back to the atmosphere (Norby and Jackson, 2000).

In order to investigate how climatic changes will affect biological processes and natural ecosystems, the CLIMAITE project (CLIMate change effects In Terrestrial Ecosystems) was set up in 2005. In CLIMAITE, the effects of CO_2 enrichment, increased temperature and summer drought on root length and distribution were investigated in a multifactor experiment in a mixed heath land/grassland ecosystem in Denmark.

We hypothesize that root growth is increased in elevated CO_2, due to extra C allocation. We also hypothesize roots to grow deeper in the soil under elevated CO_2, to sustain an increased growth in this nutrient limited ecosystem. Further we expect warming to increase growth, but only if there is no decrease in soil water content, as drought decreases root growth. Finally we hypothesize that CO_2 will counterbalance the negative effect of drought.

MATERIALS AND METHODS

Site description

The experimental site is situated in a dry heath land/grass land app. 50 km NW of Copenhagen (55∘ 53' N, 11∘ 58' E), Denmark, on a hilly nutrient-poor acid sandy deposit. The soil consists of 71.5% sand, 20.5 % coarse sand, 5.8% silt and 2.2% clay (Nielsen *et al.* 2009). The soil is well drained with a pH_{CaCl2} in the topsoil of 3.3 increasing to 4.5 in the B-horizon and an organic top layer of 2-5 cm (O-horizon).
The yearly mean temperature is 8 °C and the yearly mean precipitation is 613 mm (Danish Meteorological Institute, 2009). The site had relatively low atmospheric N bulk deposition of 1.35±0.04 g N m-2 y-1 in 2007 (Larsen *et al.* 2011).

The experimental area has been managed by sporadically cutting, removal of trees and natural grazing until 2004.

The dominant plant species are the evergreen dwarf shrub *Calluna vulgaris* (L.) Hull (c. 30 % cover) and the perennial grass *Deschampsia flexuosa* (L.) Trin (c. 70% cover).

Experimental design

The CLIMAITE manipulation experiment started in October 2005. The treatments were made to match the Danish climate scenario in 2075, as predicted by the Danish Meteorological Institute (Danish Meteorological Institute, 2009, http://www.DMI.dk), with one important exception: precipitation is forecasted to change with prolonged summer droughts and increased winter precipitation, but with no major changes in annual amounts. The CLIMAITE experiment focussed on the summer drought only, because eventual responses would be difficult to interpret in a combined summer removal and winter addition scenario.

The experiment consists of 12 octagons (7 m in diameter) laid out pair wise in 6 blocks. Each block comprises two octagons, one of which receives elevated CO_2 by FACE (Free Air Carbon Enrichment) technique and the other receiving ambient CO_2 (A). Within each octagon there are four subplots with the following treatments: summer drought (D, exclusion of rain by automatic shelters), elevated temperature (T, passive night time warming by reflective curtains), a combination of drought and elevated temperature (TD), and an untreated control for reference (A). The experiment provides a full factorial design replicated 6 times with the treatments and combinations: A, T, D, CO2, TD, TCO2, DCO2 and TDCO2, giving a total of 48 plots in a split plot design.

The elevated CO_2 plots have a target concentration of 510 ppm. The enrichment starts at dawn and continues until dusk. During night and with complete snow cover the CO_2 is turned off. The temperature treatment (T) elevates the temperature by average 0.8 degrees C in 2 cm soil depth by passive night time warming, with reflectance curtains from dusk till dawn. Drought (D) is exposed to the site once or twice every year in spring or summer time by exclusion of rain by automatic curtains. The drought continues for 2-5 weeks or until soil water content gets below 5 vol% water content in the top 20 cm of the soil. Further details of experimental setup are given in Mikkelsen *et al* (2008) and Larsen *et al* (2011).

The drought periods in 2009 were 18/5 – 25/5 and again 25/6-13/7, excluding a total of 1.6929 in of rain. In 2010, the drought was from 4/5 to 3/6 and excluded 70 mm of rain. Figure 1 shows the climatic data at the site and soil moisture conditions in control plots from 2007-2010.

Root biomass

Roots were sampled under mixed *Calluna* and *Deschampsia* vegetation in beginning of July 2007, in each experimental plot. Soil was sampled down to 70 cm with a soil auger of 6.5 cm in diameter and divided into horizons: Organic horizon (O-horizon), 0-5 cm, 5-10 cm, 10-30 and 30 to 70 cm depth. Roots were separated from the soil in the lab by use of sieves and hand picking of roots with forceps, and then washed carefully. The roots were then dried in the oven at app. 60 °C and weighed to get the dry weight (DW).

Installation of minirhizotron tubes and image collection

For image analysis of roots, we installed 48 minirhizotron tubes made of high grade acrylic in July 2007, under mixed vegetation of *Calluna* and *Deschampsia*.

One tube per plot was installed in an angle of 45 degrees to horizontal. Tube holes were cored out using a core size slightly bigger than the tube size (bought together with the root scanner from CID. Inc. USA). The tubes were 6.35 cm inner diameter and 1.1 m long and the 345º images were taken to a vertical depth of c. 50 cm. The tubes were internally insulated with foam padding/pipe insulation between imaging periods and the tubes were sealed with black rubber lids to exclude moisture and debris. The part of the tube above ground (app. 5 cm) was covered with black electrical tape to prevent any light from entering the tubes, and was covered by vegetation soon after the installation.

The image collection started in the summer of 2008, one year after installation, in order to allow time for roots to establish around the tubes prior to start of the measurements. Minirhizotron images were collected every 2-4 weeks during summer and every second month during winter from august 2008 to august 2009, and with larger intervals during the following 12 months ending in July 2010 (4 and 5 years after start of treatment). The root imaging was done by using a CI-600 root scanner (CID, Camas, Wash., USA), and colour pictures were taken at 300 dpi.

Owing to poor contact with soil near the surface, information from the first subsample was discarded, and only images from 8 cm and deeper were analyzed. Due to the large number of roots, we analysed 14 subsamples down the soil profile. All sub samples were cut out from the same location in all images, on the side of the tube, as the roots were distributed unevenly around the tube. This is in accordance with Iversen *et al.* (2011), who reported that the tube sides may be the most representative of total tube colonisation. The images were later analyzed with the free, open-source software

program Rootfly (Rootfly Version 1.8.35 Copyright [C] 2008 Clemson University).

At each sampling date we determined the total standing root length pr tube by summing all root lengths (mm) present in a window during a sampling date. Growth was defined as new root length that appeared as the result of either the growth of a pre-existing root or the growth of a new root that appeared in a window between sampling dates.

The output from Rootfly provides a 'maximum root length' and 'maximum diameter' for each individual root observed during the study, within the image area. This means that the maximum diameter or maximum length could be from any period within the year. One root might reach a max diameter at the first sampling date, while another root could reach a maximum diameter on the last sampling date within that year.

When calculating turnover, we used the turnover index: new length growth summed over periods of one year divided by total root length averaged across periods for that year (Milchunas *et al.* 2005). We calculated turnover from the last year of measurement, i.e. from July 2009 to July 2010. We were unable to distinguish among roots of the two dominant species, *Calluna* and *Deschampsia*, and thus only considered relative responses of the root system of the entire plant community.

In total 17860 images were digitized and 13476 individual roots were followed and used for analysis of the treatment effects throughout 2 years, i.e. 25 sampling dates.

Statistics

For the statistical analysis of standing biomass, maximum root diameter and maximum root length we used a mixed effects model with random effects accounting for the experimental design (*random effects of* block, octagon, octagon×D, octagon×T). We included all main effects and interactions between treatment variables drought, warming and CO_2. Model reduction was done to obtain the best model by sequential removal of the terms in the model with the highest P value. Differences of Least Squares Means (DLSM) were used to interpret significant interactions.

Repeated measurements of root length and root number over time were analysed using a mixed linear model. We found that a reasonable model for the covariance structure within subject was obtained with a spatial exponential correlation with no random intercept term. Likelihood ratio tests were used to test for treatment differences in the mean growth curves for root length and root number.

Data were transformed in case model assumptions of normality and homogeneity of variances were not met. P-values <0.05 were considered significant, but effects with P<0.1 are also reported. The statistical analyses for repeated measurements were done using "R: A language and environment for statistical Computing" while the remaining analyses were conducted using the PROC MIXED procedure of SAS (SAS Institute, 2003).

RESULTS

Standing biomass

The root biomass sampled in July 2007 after two years of treatment was highest in the 0-5 cm depth in all treatments and decreasing down the profile (Table 1). The samples were taken a few weeks after the drought treatment ended, and showed a significant interaction effect of T×D (P=0.0349) in the O-horizon. Elevated temperature and drought in combination decreased the root biomass compared to the single effects of elevated temperature or drought. The combined T and CO_2 treatment interacted to stimulate root growth in depth 5-10 cm depth (P=0.0137), where elevated CO_2 alleviated the negative effects on root biomass of T and TD treatments when in combination. The root biomass in the deeper layers did not show any significant treatment effects.

The total root biomass from O-horizon to 70 cm depth was stimulated c. 15 % by elevated CO_2 (P=0.0186), compared to ambient CO_2.

Root length

The standing root length measured by minirhizotrons increased in all treatments during the two years, especially in the first year (Fig. 2). The data suggest that the roots around the minirhizotrons had likely not reached equilibrium during the first 12 months of measurements, even though the tubes were installed in July 2007, one year prior to root scanning. This corresponds to Milchunas *et al.* (2005) and Anderson *et al.* (2010), who report of equilibrium time of more than a year, and up to 5 years.

The following year the standing root length stabilized and we therefore assume that the last year of measurement is the most representative for this ecosystem.

The total root length in the soil profile was significantly increased by elevated CO_2 (P=0.003), from January 2009 and throughout the study (Fig. 3 A). The 14 images from each minirhizotron were divided into three depth

increments (8-15 cm, 15-25 cm and 25-50 cm vertical depth) and analyzed for treatment effects.

In the upper part of the soil from 8 to 15 cm depth the warming treatment significantly decreased the root length in all combinations (P=0.030) from October 2008 and throughout the study (Fig 3B). CO_2 tended to stimulate the root length in the upper part of the soil (P=0.054) (Fig. 4A, B).

In the depth 15-25 cm, the root length tended to be reduced by warming (P=0.098) and drought (P=0.0825) and to be stimulated by elevated CO_2 (P=0.063) (Fig. 4A, B). In the deepest part of the soil, from 25 to 48 cm of depth, elevated CO_2 tended to stimulate root length (P=0.076) in all combinations from December 2008 and onwards (Fig. 4A, B).

When analysis were done for root lengths at the very last measurement in the study (almost 5 years after the onset of the experiment), the CO_2 effects were significant for the whole soil profile (+51%, P=0.024), and for all depth intervals 8-15 cm (P=0.012), 15-25 cm (P=0.015) and 25-50 cm (P=0.048) (Fig. 4 A and B). Hence the responses got significantly stronger, with duration of the study.

The 'maximum root length' and 'maximum diameter' for each individual root observed during the study were also determined (Table 2). The majority of the roots were fine roots (76% < 0.40 mm, Fig 5). However, due to low image quality, the very fine roots (< 0.2 mm) may be underestimated or missed. Mean 'max. root diameter' was similar in all treatments across the 2 years (mean = 0.34, median=0.32 max=1.07 and min= 0.16) and was not affected by the treatments over the whole soil profile. However, elevated temperature increased the root diameter in 25-50 cm depth in the last year of the observation (P=0.0448).

The maximum root length of individual roots, was increased by warming, in 25 to 50 cm soil depth (P=0.0245). There was also a significant interaction of elevated temperature and elevated CO_2 (P=0.0073), where the interaction resulted in no effect of elevated temperature, when it was combined with elevated CO_2.

Root number

Elevated CO_2 tended to stimulate total root number compared to ambient CO_2 (P=0.083) when looking at the whole soil profile (Table 2). The number of roots was similar between elevated CO_2 and ambient treatments in the upper most soil layer (8-15 cm).

There was a significant effect of warming (P=0.0386), and highly significant effects of CO_2 (P=0.0008) and drought (P=0.0002) on root number in 15-25 cm soil depth. Elevated CO_2 seemed to increase the root number in

15-25 cm soil depth while the direction of the effect of drought appeared to depend on the level of CO_2. In the lower soil depth from 25-50 cm there was a tendency towards increased root number for elevated CO_2 (P=0.084).

Fine root longevity, production and turnover

We could not accurately estimate when roots died due to lack of changes in root colour or appearance and therefore the root measure is rather persistence of roots. Only 19% of all observed roots disappeared during the 2 years of study, hence a longer study period is probably needed to get an estimate of root longevity.

The fine root production (Fig. 6) followed a seasonal pattern in 2009 with large peaks in the summer time, and from April to June the fine root production was significantly higher in elevated CO_2 compared to ambient (P<0.05 for sessions measured in April-June). No treatment effects were observed in 2010, data not shown.

Root decomposition was generally slow and after almost 3 years the mass loss of root litter in the control plots was only 39 % and 45 % in *Calluna* and *Deschampsia* respectively (data not shown), which suggest that many of the roots may have died long before they disappear.

The relative root turnover was significantly higher in elevated temperature in the last year of treatment, i.e. from July 2009 to July 2010 (Table 2).

DISCUSSION

Minirhizotrons have enabled non-destructive determination of root dynamics in time. Usually it is recommended that the tubes are installed in the soil one year prior to image collection, to reach equilibrium (Johnson *et al.* 2001) defined by the ratio between root growth and root mortality which should fluctuate between a value of 1 and have a slope of 0 (Milchunas *et al.* 2005). However, studies have reported equilibrium times of up to 4 years (Iversen *et al.* 2008) and 6 years (Milchunas *et al.* 2005) after installation, and longer equilibrium time is needed to get an accurate estimate of root growth and loss, as suggested by (Ferguson and Nowak, 2011; Strand *et al.* 2008). In nutrient limited or arid ecosystems with slow turnover longer equilibrium time might be most important. The minirhizotron data presented here provide a measure of relative differences in root production rather than absolute values for this ecosystem. The continuous increasing root length growth in the minirhizotrons suggests that equilibrium had likely not been reached. Although we measured during two years, on top of one year equilibrium time, the study may not characterize a new steady state response to climate change, but rather a transition period.

CO_2 response

Elevated CO_2 stimulated root biomass (after 2 years of treatments) and total root length (after 4 and 5 years of treatment), measured by both soil coring and minirhizotrons, as we expected. Elevated CO_2 has been shown to stimulate root length (Higgins *et al.* 2002; Milchunas *et al.* 2005; LeCain *et al.* 2006) and root biomass (Fitter *et al.* 1997) in grasslands and steppe. The observed CO_2 effect was significant in both the total biomass pool and the total standing root length, although the increase in biomass after exposure to elevated CO_2 for two years was smaller than the response in root length after 5 years of treatment. For the biomass, new growth was only slowly being added to the existing, large biomass pool, and 2007 was only the second year of the experiment, which might explain why the response was lower than measured by minirhizotrons later in the experiment.

In minirhizotrons elevated CO_2 increased both root number (+53%) and root length (+45%) at the end of the study, which suggests similar treatment response for these two. Johnson *et al* (2001) report that root length is a more sensitive metric for dynamic root properties than the root number. The greater standing root lengths was related to greater number of roots under elevated CO_2 rather than longer individual roots, as also reported by Milchunas *et*

al (2005). The difference in root length by 45% of elevated compared to ambient CO_2 corresponds to other studies in grasslands of +37% (Volder *et al.* 2007), +37 % (LeCain *et al.* 2006), in steppe +52 % (Milchunas *et al.* 2005), and forest +23% (Pritchard *et al.* 2008).

The general increase in root length and root number in elevated CO_2 is probably a response to higher plant nutrient demand. The extra root length is a way to get access to more nutrients by exploring a larger soil volume. However growing too many roots may also be undesirable, as root growth requires C and nutrients that could otherwise be allocated to photosynthetic organs (Eissenstat and Yanai, 2002). Interestingly, except for reproductive structures no persisting changes in aboveground plant biomass were observed (Kongstad *et al.* 2011), and most of the extra carbon assimilated (Albert *et al.* 2011a; Albert *et al.* 2011b) seems to be allocated belowground as a response to elevated CO_2 in this heathland.

Temperature response

We hypothesized that warming increased root growth, but the opposite effect was seen in the upper part of the soil (8-15 cm) where warming reduced the root length, measured by minirhizotrons. There could be two explanations for this, the first being increased nutrient availability. According to the 'functional equilibrium' model, plants respond to a decrease in belowground resource availability by an increased allocation to roots (Poorter and Nagel, 2000), and therefore an increased nutrient availability may lead to lower below ground biomass. Hence, a lower root production could be ascribed to a higher nutrient content in soil, perhaps due to higher mineralization and availability of nutrients. The short term results at the CLIMAITE site support the hypothesis of higher mineralization in warmed plots (Andresen *et al.* 2010; Larsen *et al.* 2011). Higher mobilization of N in top soil after warming has also been observed in other temperate heathlands (Schmidt *et al.* 2004), and in sub-arctic heaths (Schmidt *et al.* 1999; Schmidt *et al.* 2002).

The second explanation could be decreased water availability, as continuous warming leads to lower soil water content. The effect of elevated temperature on aboveground plant parts seems to appear in the beginning of the growing season with earlier onset of growing season (Albert *et al.* 2011a) and a higher plant biomass (Kongstad *et al.* 2011).The earlier start of growing season in response to warming increased water consumption and reduced water availability, net photosynthesis and growth (in 2006) (Albert *et al.* 2011a) and therefore potentially reduced the absolute amount of C allocated belowground. It is reported that warming may stimulate evapotranspiration leading to a decrease in soil water content (mainly in arid ecosys-

tems), which indirectly suppress root production and increase mortality (Adair *et al.* 2009). Together the indirect or resulting effects of elevated temperature could explain the negative effect seen on root growth. Decreased annual root production, mortality and mean standing crop was also observed in temperate steppe in China in response to experimental warming (Bai *et al.* 2010), and warming also reduced the root mass in mixed swards (Lilley *et al.* 2001).

Elevated temperature had a positive effect on root diameter and root length in the deeper part of the soil, suggesting that these deeper roots are longer lived transportation roots used for water uptake, rather than nutrient uptake. A strategy for roots to cope with low soil moisture is to explore deeper parts of the soil profile (Reid and Renquist, 1997).

Drought response

The results from the minirhizotrons show significant negative effects of drought on root number, and tendency for negative drought effects on root length in 15-25 cm. However, the negative effect disappeared when drought was combined with elevated CO_2, which suggests that in this dry heathland CO_2 alleviated the drought effect due to better water use efficiency, as expected.

The drought effects on root number persisted throughout the year, contrary to the aboveground biomass where there was only a transient response to drought in year 2006-2008 (Kongstad *et al.* 2011). Both species recovered fast after rewetting and the drought had no significant effect on annual aboveground biomass production.

From the biomass harvest we saw a negative effect on the root biomass when drought and elevated temperature were combined in the O-horizon, and 5-10 cm. The O-horizon is the most drought exposed horizon, and warming may stimulate evapotranspiration and decrease the soil water content even further. Overall, treatment TD had the lowest soil water content in the summer of 2007 (0-60 cm depth, data not shown), and as the soil samples were also taken shortly after the drought period ended, this explains the negative effect of TD on root biomass.

Root distribution

The root distribution changed under elevated CO_2 to increased root length and more roots deeper in the soil profile. Mining for nutrients is one of the main reasons for greater root proliferation in deeper soils under elevated CO_2

(Iversen, 2010). Nutrient availability in shallower soil is limited as a result of increased microbial or plant competition and the extra C under elevated CO_2 can be allocated to roots growing deeper. The 'extra' N source may come from a greater cumulative amount of N available at depth – i.e. a larger pool of N when deeper soil depths are considered (Iversen, 2010). The N uptake from the soil showed sustained increase in response to elevated CO_2 in forests (Finzi *et al.* 2007). Studies of N uptake at our site showed that the increased root growth is not matched by an increased N uptake and hence the root N concentrations were decreased under elevated CO_2 (Arndal *et al*, submitted).

The question raised by Anderson *et al.* (2010) was whether the extra root biomass under elevated CO_2 will enter the recalcitrant soil organic matter pool or be recycled relatively rapidly in the soil. Previous studies have shown that additionally belowground carbon input mainly entered the fast cycling carbon pool and contributed little to the long term carbon storage under elevated CO_2 (Kandeler *et al.* 2006), also confirmed by a nine years FACE study where soil carbon sequestration in well-maintained older temperate grasslands was limited (Xie *et al.* 2005). An explanation could be that the increased root proliferation deeper in relatively unexplored soil under elevated CO2 may affect previously stable organic matter (SOM) pools in the deeper soil (Iversen, 2010). Belowground C allocation through roots may stimulate microbial community and enhance the rates of soil organic matter decomposition in the rhizosphere (Kuzyakov *et al.* 2007), a process referred to as the 'priming effect'. This was seen in a grassland study, where labile soil C increased under elevated CO_2 and set off the loss of older mineral-associated organic matter (Gill *et al.* 2002). The energy gained from these fresh inputs of labile C and N compounds from root exudation (de Graaff *et al.* 2007) or detritus from root turnover may be more important than temperature and soil moisture in stimulating the decomposition of ancient C deeper in the soil (Iversen, 2010). Increased C and N input into the soil, especially below 30 cm depth, might alter the C storage and N mineralization (Iversen *et al.* 2008). Carbon flux measurements at our site showed increased C efflux under elevated CO_2 (Selsted *et al.* 2012) exceeding the increased NEE, suggesting a priming effect of older carbon deeper in the soil. However, this was shown after 2 years of treatment, and the question is if this is a transient response. After a period of time a new equilibrium will probably be reached between C input and output belowground. After nine years, the CO_2 effect on root productivity was no longer present in a shrub-oak ecosystem (Stover *et al.* 2010), and longer term studies are needed to give valid conclusions regarding root dynamics and C balance in a future climate.

Turnover

One of the objectives of this study was to follow the lifespan and death of individual roots and to get an estimate of root turnover. However, visual separation of live and dead roots in minirhizotrons is often not possible (Valenzuela-Estrada *et al.* 2008) potentially leading to overestimation of the standing crop of roots, as some imaged roots counting as alive may actually be dead. A combined live and dead category is what is frequently measured (Johnson *et al.* 2001). The root turnover estimate should therefore be viewed with caution, due to the fact that standing crop might include dead roots. Hence calculated turnover is probably lower than real turnover, and is just a relative measure between the 8 treatments in this ecosystem. However the relative differences of turnover among treatments are valid and might explain some of the observations.

Turnover rates of 0.06-0.11 year^{-1}, similar to our estimates, were found in a desert ecosystem (Ferguson and Nowak, 2011), but another heathland study found root turnover of 0.64 and 0.96 for *Calluna vulgaris* and *Deschampsia flexuosa* roots, respectively (Aerts and Heil, 1993). Our low turnover may be due to an overestimate of living root length and a very low mortality. This pattern has also been found in other heath land studies, with a continuous increase in living root lengths during three years of observation in *Dechampsia*, due to low mortality (Aerts *et al.* 1992). A low turnover of *Calluna* roots is also expected, and can be explained as a nutrient conservation mechanism, as *Calluna* is a dominant species of low-nutrient heath lands (Aerts *et al.* 1992; Aerts and Chapin, 2000).

Our results indicate no effects of elevated CO_2 on root turnover, corresponding to Volder *et al.* (2007), who found that elevated CO_2 did not appear to alter the turnover of grasses, despite an increased allocation belowground. As elevated CO_2 are predicted to decrease the root N concentration and reduce the root maintenance respiration, slightly longer lifespan are expected (Eissenstat *et al.* 2000).

We did however find an increased root turnover in elevated temperature. This is consistent with Gill and Jackson (2000) who reported exponential increases in root turnover with mean annual temperature for grassland fine roots, at a global scale. Temperature has a strong role in determining root turnover, through its controls on root respiration and nitrogen mineralization, and further the onset of growing season is often dependent on soil temperature in spring (Gill and Jackson, 2000). Our warming treatment is a nighttime warming, and across the year the largest warming occurs in the autumn and early spring. The growth potential (Growing Degree Days) increased significantly in the early spring with a 33% higher accumulated GDD during the period from 1 April to 15 May 2006 (Mikkelsen *et al.* 2008). The longer

growing season and increased maintenance respiration in warmer soils can explain the observed higher turnover.

In summary, the minirhizotron data indicates that 2 years after tube installation, the roots around the tube might still not be in equilibrium. This should be kept in mind, when nutrient poor or slow growing ecosystems are planned to be studied with minirhizotrons.

In the future climate scenario (TDCO2) the strong response of elevated CO_2 led to increased root length deeper in the soil. This shift in rooting patterns might also have great implication for the C balance, dependent on whether the extra root biomass will enter the recalcitrant soil organic matter pool or be recycled relatively rapidly in the soil. The increase in fine root production could ultimately result in increased C sequestration in this ecosystem, due to the very low root decomposition observed.

As no aboveground plant composition changes are reported from the site, this suggests that the ecosystem might be in a transition period, where the greater exploitation of deeper soil layers is a response to limited nutrients. This essentially might result in changes in the aboveground biomass and species composition as well.

Acknowledgements

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TABLES

Table 1. *Standing root biomass (g m^{-2}), mean± SE for July 2007. Treatments were A: ambient, T: elevated temperature, D: drought, CO2: elevated CO2 and the combinations TD, TCO2, DCO2 and TDCO2.*

Soil depth cm	A	T	D	TD	CO2	TCO2	DCO2	TDCO2	P values
O-hor.	63±23	84±37	101±28	59±23	73±23	187±58	136±35	53±15	T×D: 0.0349
0 til 5	245±52	167±29	242±43	148±20	225±34	185±24	179±44	194±17	
5 til 10	120±15	82±17	101±18	79±9	105±11	132±23	107±26	113±12	T×CO2: 0.0137
10 til 30	123±28	77±13	80±13	90±32	85±7	76±14	90±18	115±16	
30 til 70	46±17	36±5	55±15	46±17	94±29	50±18	76±19	68±23	

Table 2.*'Max root diameter' and 'max root length' (in mm) of individual roots (mean±SE) calculated for the second year of observations in 25-50 cm depth. There were no significant effects in the upper parts of the soil. Root number per tube (mean± SE) across the whole soil profile observed in the second year. Turnover across all soil depths was calculated from the second year of measurements as: New root length growth (yr)/mean total root length (yr) = turnover year^{-1}. Treatments were A:Ambient, T: elevated temperature, D: Drought, CO2: elevated CO$_2$ and the combinations TD, TCO2, DCO2 and TDCO2.*

A	T	D	TD	CO2	TCO2	DCO2	TDCO2	P value
Max diam.								
0.37±0.02	0.43±0.06	0.36±0.03	0.38±0.02	0.37±0.05	0.40±0.1	0.38±0.01	0.37±0.04	T: 0.0448 T×D: †
Max length								
7.1±1.1	10.1±3.2	6.3±1.7	7.6±1.2	7.5±0.8	7.0±0.9	7.1±0.8	7.1±0.8	T: 0.0245 T×CO2: 0.007
Number tube^{-1}								
251±42	159±58	215±47	260±34	314±36	320±63	312±52	285±44	CO2: †
Turn over (year^{-1})								
0.04±0.03	0.08±0.04	0.06±0.04	0.07±0.01	0.04±0.02	0.07±0.03	0.07±0.02	0.06±0.04	T: 0.0501

FIGURE LEGENDS

Fig. 1. Average precipitation (mm), air temperature (°C) and soil water content (vol %) in 2007-2010 in control plots (treatment A).

Fig. 2. Fine root standing crop (mm/ tube image area) from July 2008 through July 2010 quantified with minirhizotrons. Error bars are omitted for figure clarity, but see Fig 3B. The treatments were A: ambient, T: elevated temperature, D: drought, CO2: elevated CO2 and the combinations TD, TCO2, DCO2 and TDCO2.

Fig. 3 A and B. The A figure shows average profiles and 95 % confidence intervals for the means for the total root length in ambient and CO2-enriched plots during the period from July 2008 to July 2010. Significantly longer root lengths (p=0.003) were found in plots with elevated level of CO2 from January 2009.
The B figure shows average profiles and 95 % confidence intervals for the means for the total root length in the upper soil depth (8-15 cm), in ambient and warming. Root lengths was significantly decreased (P=0.030) in plots with elevated temperature from October 2008 and throughout the study.

Fig. 4 A and B. The left panel displays the average root length in different parts of the soil for plots with ambient CO2 (8-15 cm, 15-25 cm and 25-50 cm soil depth). The right panel shows the average root length for plots with elevated level of CO2. At the end of the study significantly longer root lengths were observed for the whole soil profile (p=0.024) and in each soil layer (p=0.012/0.015/0.048 for upper/medium/lower layer).

Fig. 5. Distribution of fine root diameter measurements over the 2-year observation period (2008-2010) across all treatments.

Fig. 6. Average root length production are shown for plots with ambient and elevated CO2, measured as mm growth of root length per day divided by the area of the image. Though measures of increment were associated with a large sampling error significantly larger increments were observed for CO2 enriched plots at various sampling points throughout the summer of 2009.
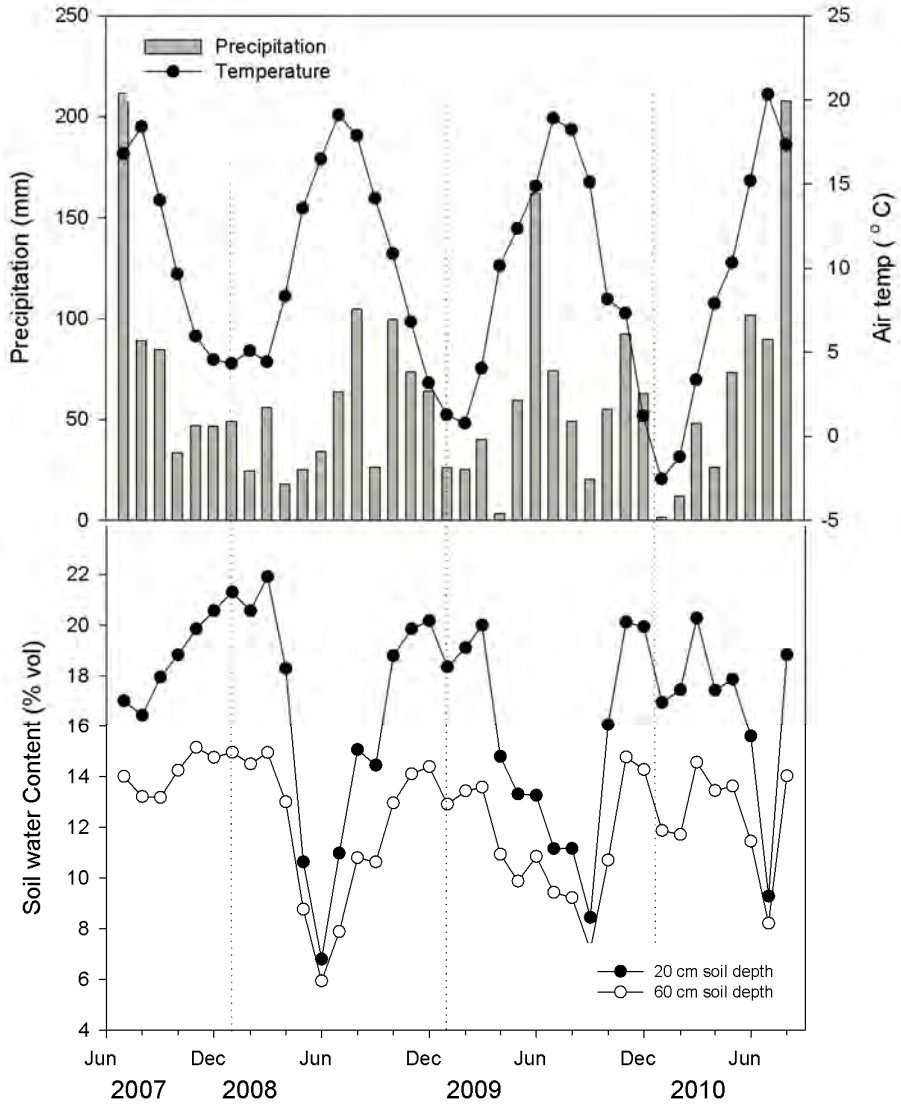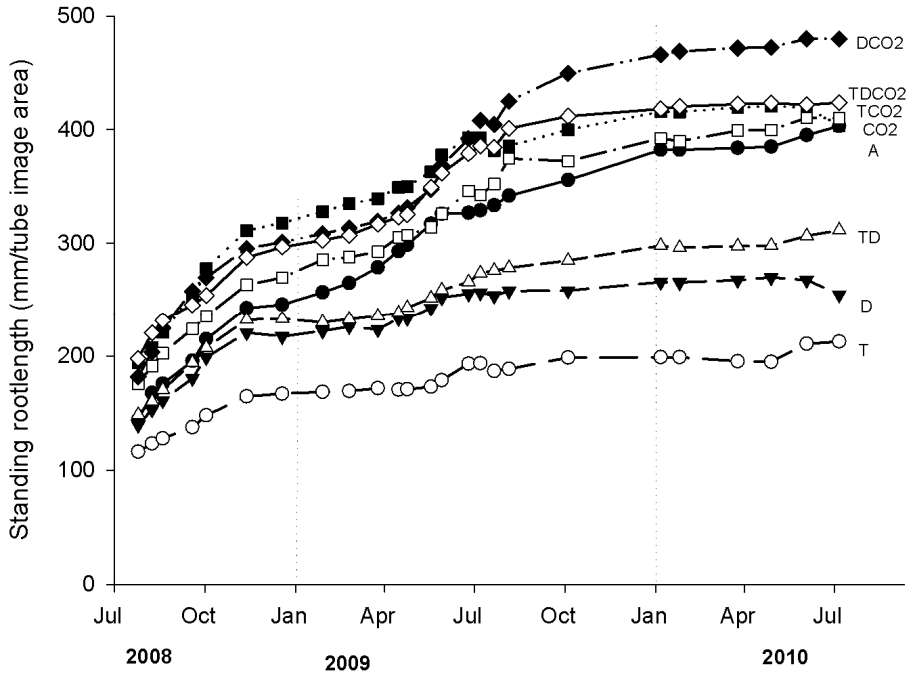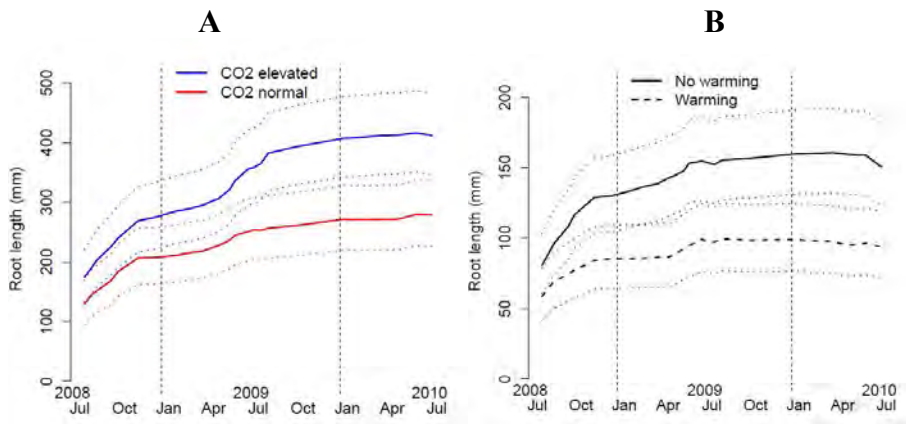
Fig.1

Fig. 2.

A

B
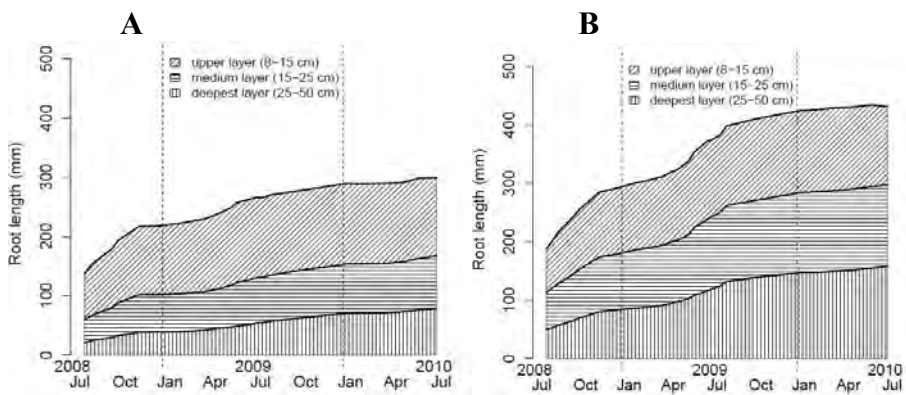

Fig. 3 A and B

A
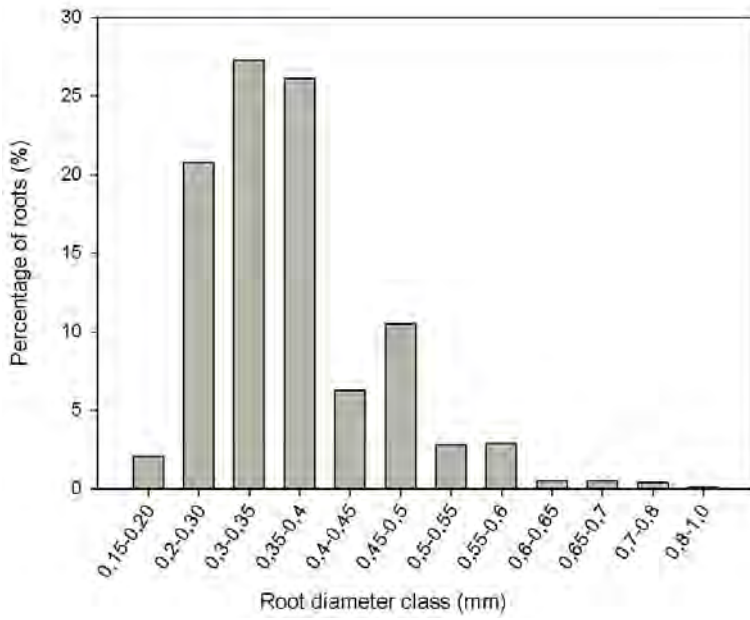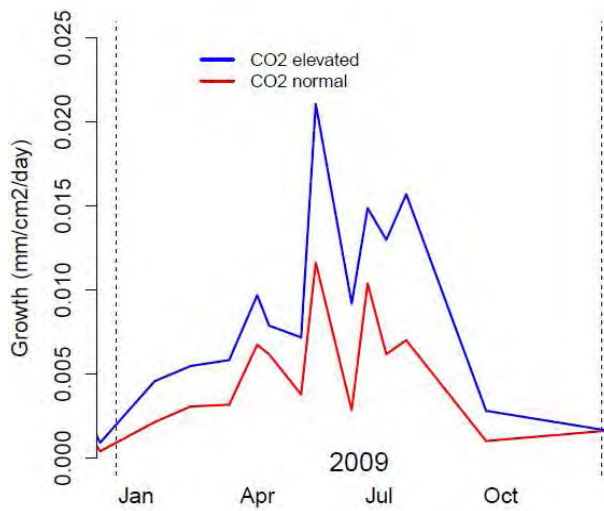
B

Fig 4 A and B.

Fig 5.

Fig. 6.

PAPER II

FINE ROOT GROWTH AND N DYNAMICS IN RESPONSES TO CLIMATE CHANGE: RESULTS FROM THE CLIMAITE EXPERIMENT

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ABSTRACT

Ecosystems exposed to elevated CO_2 are often found to sequester more atmospheric carbon, due to increases in photosynthesis, resulting in increased plant growth.

We exposed a Danish heath ecosystem to the future climate scenario in order to study if an expected increased growth would be matched by an increase in root nutrient uptake. We investigated how root growth and nutrient uptake of NH_4^+-N and NO_3^- -N was affected by elevated CO_2, elevated temperature and extended summer drought alone and in all combinations.

Root growth was significantly increased by elevated CO_2. The roots, however, did not fully compensate the higher growth by a similar increase in nutrient uptake, as the roots were unsuccessful in translating additional carbon uptake into increased nitrogen uptake per unit root. Hence the nitrogen concentration in roots was decreased in elevated CO_2, whereas the biomass N pool was not lower than ambient CO_2. The increased root growth in elevated CO_2 might be a strategy for the plants to cope with increased nutrient demand leading to a long term increased N uptake on a whole plant basis, despite unchanged N uptake per unit root mass. Drought reduced grass root biomass and N uptake, especially when combined with warming, but CO_2 was the most pronounced main factor effect. Significant second and third order interactions with warming and drought were plenty, showing that main factor effects were often not additive, and that changes to multiple environmental changes cannot be predicted from single factor responses alone.

INTRODUCTION

Most climate change scenarios project that greenhouse gas concentrations will increase over the next decades with a continued increase in average global temperatures (IPCC, 2007). Along with increasing CO_2 concentrations and a projected warming of about 0.2°C per decade, changes in precipitation patterns are also expected. These three factors are drivers for many important ecosystem processes, and changes in ecosystem function are therefore expected.

Elevated CO_2 is expected to increase plant growth, but this increase in biomass may not be sustained in the long term if nutrients are limiting. The Progressive Nitrogen Limitation (PNL) hypothesis suggests that without additional N input or reduced N loss, the N availability decreases over time at elevated CO_2 (Luo *et al.* 2004), leading to reduced C uptake in the long term. A substantial amount of carbon assimilated by plants is transported below-ground and transferred to the soil, and in temperate grasslands the largest fraction of total ecosystem carbon is stored belowground. Calculated root surface area and fine root length is often greater than leaf area, and in grasslands it is more than an order of magnitude larger (Jackson *et al.* 1997). Hence soils are often the critical C sink in grasslands, in contrast to the large aboveground biomass sink in forests (Gill *et al.* 2006). Under elevated CO_2 plants show increased water use efficiency and rate of photosynthesis (Anderson *et al.* 2010; Rogers *et al.* 1994), and more carbon is often allocated to the roots (Fitter *et al.* 1997; Higgins *et al.* 2002; VanVuuren *et al.* 1997). Major functions of plant roots are absorption of water and inorganic nutrients. Regulation of the ability to take up nutrients, such as nitrogen (N), might have great influence on carbon sequestration (Bassirirad, 2000), and nutrient uptake kinetics are expected to change under elevated CO_2. This is due to 1) higher availability of carbohydrates under elevated CO_2 which may result in up-regulation of root nutrient transporters, 2) elevated CO_2 accelerates growth and hence increase plant nutrient demand and uptake capacity (Bielenberg and Bassirirad, 2005).

The root N uptake rates are expected to be stimulated under high CO_2 (Jackson and Reynolds, 1996). Especially NO_3^--N uptake is expected to be positively affected (Bassirirad *et al.* 1999, 1996), due to the increased carbohydrate levels in roots in elevated CO_2, which may support the energy demanding nitrate reductase activity in roots. For example, Jin and Evans (2007) found increased NH_4^+-N and NO_3^--N uptake under elevated CO_2 in arid shrub lands, with NO_3^- derived N taken up at greatest rates.

Species specific changes in the root capacity for N uptake can also influence competitive interactions between species and vegetation dynamics as well as

N cycling (Bassirirad *et al.* 1996). If plant growth is stimulated under elevated CO_2 but the absolute nutrient uptake is not affected, C/N ratio would increase at the whole plant level (Gifford *et al*. 2000) which would reduce litter quality (Cotrufo *et al.* 1998; Gill *et al.* 2006).

Together with mineralization, diffusion and mass flow, soil temperature determine nutrient uptake (Pregitzer and King, 2005). Soil temperature directly affects root physiology in several ways by altering the specific rates of ion uptake, root respiration, cell membrane permeability etc. Nutrient uptake capacity generally increases in response to increasing temperatures – although caution should be made about generalization across a broad range of species and soil temperatures (Bassirirad, 2000). Increasing soil temperature stimulates root growth, when all other factors remain equal. This can be due to increases in photosynthesis with higher temperatures, where the extra fixed carbon is allocated belowground to sustain new root growth (Pregitzer and King, 2005).

Increasing soil temperatures can also stimulate the production of growth relating substances (e.g. ABA, gibberellins). Often increasing temperatures are followed by increased evapotranspiration, leading to drought conditions. When water is not limiting, roots proliferate near the surface of the soil where higher temperatures do not limit growth and accelerate rates of nutrient mineralization (Pregitzer and King, 2005). Consequently low soil moisture reduces nutrient availability and nutrient uptake in the rooting zone and thus decreases uptake rates (Gutschick and Pushnik, 2005). Dry soil prevents mass flow and diffusion of nutrients, but can also lead to increased mechanical impedance to root growth and thereby limit nutrient interception (Pregitzer and King, 2005).

The direct effects of warming and CO_2 on plant growth in natural ecosystems can be limited if nutrient limitation constrains the carbon allocation to growth (Arnone *et al.* 2000; Gill *et al.* 2006). This may be the case in already nutrient limited ecosystems, but may otherwise occur over longer periods of time if nutrient stocks are gradually depleted (Pregitzer and King, 2005), leading to progressive nitrogen limitation, where available soil N becomes increasingly limiting as C and N are sequestered in long living biomass and soil organic matter (Luo *et al.* 2004). PNL is less likely to occur if the increased N demand can be met by additional supply and several examples exist with no evidence of progressive nitrogen limitation even after several years of treatments with elevated CO_2 treatments in forests (Barnard *et al.* 2006; Norby and Iversen, 2006; Phillips *et al.* 2006).

Studies of potential changes in root uptake capacity in response to the projected climate changes are few and inconsistent. This is despite the recognition, that plant responses to CO_2 are very dependent on plant N acquisition. It is critical to know, if the plants can positively adjust to the expected

increased demand of nitrogen under elevated CO_2 by increasing the root growth or physiological properties.

Although plant uptake of glycine was studied at our experimental site one year after treatments started (Andresen *et al.* 2009), there are no long-term investigations of effects of combined elevated CO_2, temperature and drought on root dynamics and root-plant-nutrient interactions. Here we report the response of root growth and uptake of ammonium (NH_4^+-N) and nitrate (NO_3^--N) to changes in atmospheric CO_2, temperature and prolonged summer drought in all combinations.

We hypothesize the following:

- Root growth is stimulated in response to elevated CO_2 due to increased C uptake and therefore greater allocation of C to roots. Drought is expected to slow down root growth, while the temperature treatment will increase root growth, depending on soil moisture content.

- Nitrogen uptake will increase in response to elevated CO_2 due to a higher demand for nutrients to sustain increased growth. N acquisition will increase due to higher mineralization in warming, while drought by contrast is expected to decrease the N acquisition.

- Increased root growth by elevated CO_2 reduces root nitrogen concentration due to higher increase in photosynthetic assimilation of carbon relative to plant N uptake.

MATERIALS AND METHODS

Site description

The experimental site is situated in a dry heath land/grass land app. 50 km NW of Copenhagen (55° 53' N, 11° 58' E), Denmark, on a hilly nutrient-poor acid sandy deposit. The soil consists of 71.5% sand, 20.5 % coarse sand, 5.8% silt and 2.2% clay (Nielsen *et al.* 2009). The soil is well drained with a pH_{CaCl2} in the topsoil of 3.3 increasing to 4.5 in the B-horizon and an organic top layer of 2-5 cm (O-horizon).

The yearly mean temperature is 8 °C and the yearly mean precipitation is 613 mm (Danish Meteorological Institute, 2009). The site had relatively low atmospheric N bulk deposition of 1.35±0.04 g N m^{-2} y^{-1} in 2007 (Larsen *et al.* 2011).

The experimental area has been managed by sporadically cutting, removal of trees and natural grazing until 2004. The dominant and studied plant species are the evergreen dwarf shrub *Calluna vulgaris* Hull. (c. 30 % cover) and the perennial grass *Deschampsia flexuosa* Trin. (c. 70% cover). Few other grasses, herbs and mosses common for acidic grassland are also present at the site.

Experimental manipulations

The manipulations in the CLIMAITE experiment are designed according to the climate predictions for Denmark in year 2075. The climate scenario is elevated atmospheric CO_2 concentration at 510 ppm, elevated temperatures of 1-2 °C and a prolonged summer drought period (4-6 weeks).

The experiment consists of 12 octagons (7 m in diameter) laid out pair wise in 6 blocks. Each block consists of two octagons with one octagon receiving elevated CO_2 (CO_2, 510 ppm) by FACE technique and the other receiving ambient CO_2 (A). Within each octagon there are four subplots with the following treatments: summer drought (D, exclusion of rain by automatic shelters), elevated temperature (T, passive night time warming by reflective curtains), a combination of drought and elevated temperature (TD), and an untreated control for reference (A) in a split plot design.

The experiment provides a full factorial design replicated 6 times with the treatments and combinations: A, T, D, CO2, TD, TCO2, DCO2 and TDCO2, giving a total of 48 plots. The treatments were initiated in October 2005. See Mikkelsen *et al*. (2008) and Larsen *et al*. (2011) for further details on experimental design and set up. Figure 1 shows the climatic data at the site and soil moisture conditions in control plots from 2007-2008.

The imposed drought periods were in 2007: 5/5 to 27/5, 2008: 5/5 to 27/5 and again 16/9 to 2/10. There was only a minor exclusion of rain in 2008 due to an extremely dry summer, while in 2007 94 mm of rain were excluded.

Root biomass

Soil samples were taken in October 2008 to give an estimate of standing root biomass under both *Calluna* and *Deschampsia* vegetation, in each experimental plot. The soil auger was 4.5 cm in diameter and the soil core was divided into horizons: O-horizon, 0-5 cm, 5-10 cm depth as for the in-growth cores. Roots were separated from the soil in the soil laboratory by use of sieves and hand picking of roots with forceps, and then washed carefully. The roots were dried in the oven at app. 60 °C and weighed.

Root in-growth core and N pool size estimation

Relative root growth was estimated by in-growth cores, covering a full year of root growth from October 2007 to November 2008. In each treatment plot, two soil cores were sampled under patches of *Calluna* and *Deschampsia*, respectively. Roots were separated from the soil by use of sieves and picking of roots with forceps. A tube of plastic mesh (mesh size app. 2 mm) lined the soil pit to mark the original hole of 6 cm in diameter and this was then filled up with root free soil in the same original order of depth, divided into O-horizon, 0-5 cm and 5-10 cm depth, separated from each other by a small cloth inside the bag. After 13 months (November 2008), a soil auger of 4.5 cm was used to take a sample inside the original hole. The 96 samples were brought back to the lab and put into a fridge until time of root sorting. The roots were not separated into species, but we assumed that the majority of the roots would belong to the species under which the in-growth cores were placed due to the species patchy distribution.

In an earlier in-growth core study we found that some roots from *Deschampsia* grew into the meshbags of *Calluna* (especially in the O-horizon), while the opposite was rarely seen. A mean estimate of *Deschampsia* roots growing into *Calluna* meshbags indicated approximately 20 % grass roots in the O-horizon, 12 % in 0-5 cm and 15 % in 5-10 cm depth under the dwarf shrubs, but little or no in-growth of *Calluna* roots into meshbags under *Deschampsia* (unpublished results). Due to this, we write 'under' *Calluna or Deschampsia*, as further separation of species was not done, assuming that the majority of the root samples taken belonged to the aboveground plant species right above.

The standing pool of belowground plant nitrogen was calculated as the root biomass from the in-growth core multiplied by the nitrogen concentrations.

Nitrogen uptake

The study of nitrogen uptake was done by ^{15}N assay (Jones *et al.* 1991), and used roots from the in-growth cores. Hence, all the roots had been exposed to the experimental treatments which had been running for three years, and the roots had a maximum age of 13 months.

Due to the large number of samples and the time demanding root sorting procedure, sampling was done sequentially in 3 campaigns in autumn 2008 (two blocks = four octagons per campaign: 24th of November, 30th of November and 7th of December). The soil samples were separated into O-horizon, 0-5 cm and 5-10 cm. Each horizon was sorted by hand and fine

roots were washed in demineralised water. The roots were put in a plastic bag with a damp/moist hand towel cloth and kept dark at 5 °C until the assay was conducted.

The N bioassay was performed using the method described by Jones *et al.* (1991). The fresh roots from each species and soil layer were divided into two sub samples for analysis of absorption of $^{15}NH_4^+$-N and $^{15}NO_3^-$ -N, respectively. The root samples from the O-horizon were very small and therefore sufficed for $^{15}NH_4^+$-N uptake assay only. All roots of both *Deschampsia* and *Calluna* were approximately of same diameter (< 1 mm in diameter) and were not separated into different size classes. Hence all roots were considered active and able to take up nutrients.

The fresh root bundles were marked with a name tag and pre-soaked for 30 mins (5 $\times 10^{-4}$ M $CaCl_2$ solution) to maintain root cell membrane integrity and to remove ammonium, nitrate and nitrite ions from the cell-free space (Rosengren *et al.* 2003). After pre-soak the roots were transferred to the uptake solution (5 $\times 10^{-4}$ M $CaCl_2$ labelled with 2 ml ^{15}N-NH_4Cl/- KNO_3 and 8 ml of ^{14}N-NH_4Cl/- KNO_3, to get a 20% enrichment of ^{15}N) and soaked for 2 hours, washed in running demineralised water for 15 minutes, put in paper bags, dried in the oven at 80 °C, ground and weighed and finally analysed for ^{15}N, % N and % C (Eurovector elemental analyzer, (Milan, Italy) coupled to an Isoprime mass spectrometer (Cheadle Hulme, UK)). Excess ^{15}N (atom %) was converted to absorption rate of N (in μg N/ g root DW / 2 h). The total N concentration was also calculated, with subtraction of N that had been taken up during the bioassay (Michelsen *et al.* 1999).

The nitrogen deficiency test was developed as a tool to relate root nitrogen levels to the fertilizer regime of trees, in excised roots (Jones *et al.* 1991). High ^{15}N uptake demonstrates nitrogen limitation (Jones *et al.* 1991; Rosengren *et al.* 2003). The results obtained with this method with excised roots represent a relative measurement of the root *net* uptake capacity at different soil depths and not the actual uptake rate as would have been found with the roots still attached to the plant (Goransson *et al.* 2007).

Statistical analysis

Statistical analyses were conducted by using the PROC MIXED procedure of SAS (SAS Institute, 2003). The statistical model chosen included a random statement that accounted for the experimental design (*Random* block octagon octagon×D octagon×T). The same model was used for all variables, with all main factor effects and their interactions, except when differences between soil depths were studied. Soil sample ID was also included in the random statement to account for possible correlation between biomasses at

different depths in the same soil sample. Data were transformed in case requirements for normality and homogeneity of variances were not met. P-values <0.05 were considered significant, but effects with P<0.1 are also reported. Model reduction was done to obtain the best model by sequential removal of the terms in the model with the highest P value. Differences of Least Squares Means (DLSM) were used to interpret significant interactions.

RESULTS

Fine root biomass and relative root growth

The total root biomass of *Deschampsia* sampled in October was significantly lower in the drought treatment whereas CO_2 tended to increase the root biomass (Fig. 2). We observed no effect of any treatment on the root biomass of *Calluna*.

The effect of elevated CO_2 on *Deschampsia* root biomass was strongest in the top soil (0-5 cm, P=0.0438), and tended to increase the biomass in 5-10 cm in both species (P<0.1). Further, *Deschampsia* roots were negatively drought affected in the O-horizon (P=0.0305). In 0-5 cm there was further an interaction of T×D×CO_2 (P=0.0388), where evaluation of the three-way-interaction by DLSM showed that the biomass was decreased in TD treatment, while the full combination of TDCO_2 was higher.

CO_2 stimulated the total root growth of both plant species in the in-growth cores, with 28 % in *Calluna* and by 55 % in *Deschampsia* compared to non-CO_2 treated plots (Fig. 3). The elevated CO2 response was significant or tended to be in both species and in all three soil depths. Further there was a negative T×D interaction, due to a lower biomass when warming and TCO$_2$ was combined with drought in *Deschampsia*. This was mostly due to a negative effect of drought in the O-horizon in *Deschampsia* (D P=0.0456 and T×D P=0.0193).

Root nitrogen concentration and N pool sizes

Elevated CO_2 significantly reduced the fine root N concentration in almost all depths and under both species (Fig. 4), on average by 8.7% and 9.1 % in *Calluna* and *Deschampsia* roots, respectively (mean of all 3 depths). The root N concentration decreased with increasing soil depth.

The C/N ratio increased correspondingly in *Calluna* roots (0-5 and 5-10 cm depth, P=0.0025 and 0.0032, data not shown) and tended to increase in *Deschampsia* roots.

The root N pool size was increased in elevated temperature in *Deschampsia* O-horizon, but when combined with drought the N pool decreased (T×D interaction) (Table 1). Elevated CO_2 stimulated the root N pool size in *Deschampsia* 0-5 cm and tended to do so in 5-10 cm depth, and in *Calluna*, in the organic horizon. Hence, overall root allocation of N was increased per unit area by elevated CO_2 while the root N concentration was reduced.

Nitrogen uptake

The highest uptake of NH_4^+-N in *Calluna* roots was found in 0-5 and 5-10 cm depth, significantly higher than in roots from the O-horizon (Fig. 5). In *Deschampsia* roots the uptake in 0-5 cm depth was highest. The uptake of NH_4^+-N was similar in the two species, and with few effects of treatment. Drought reduced the uptake of NH_4^+-N in *Deschampsia* roots especially in the deeper layers (5-10 cm, P=0.0141), while CO_2 interacted with warming or TD and increased the uptake (T×CO_2, P=0.0465). The 3-factor interaction T×D×CO_2 (P=0.0306) was explained by low uptake in the TD and DCO_2 treatment, while uptake was unchanged in the full combination (TDCO_2). The uptake of NH_4^+-N in *Deschampsia* roots in the treatment TD was generally low in all three depths (non-significant).

The NH_4^+-N uptake was 3-4 times larger than the NO_3^--N uptake, and in the same order of magnitude in the two species.

The NO_3^--N uptake showed a similar pattern to the NH_4^+-N uptake in the treatments (Fig. 6). *Calluna* roots did not show any significant treatment effects in uptake, but in *Deschampsia* drought reduced or tended to reduce the uptake. In both *Calluna* and *Deschampsia* roots the uptake in the treatment TD was generally low in both depths as for NH_4^+-N uptake, non-significantly however. Roots of plants exposed to drought or a combination of drought and temperature had less N demand. However, elevated CO_2 seems to increase the demand in these treatments, especially in *Deschampsia*.

DISCUSSION

Relative root growth and root biomass

Jackson *et al*. (1997) estimated that as much as 33% of global annual net primary productivity is used for the production of fine, short lived roots. Further they estimated that global fine root carbon is more than 5% of all carbon contained in the atmosphere. Having this in mind, root responses to climate change will have great influence on the global carbon budgets.

We expected an increased root growth response to elevated CO_2. A CO_2 response was seen in our experiment, in both in-growth core and root biomass, especially in the grass *Deschampsia*. Elevated CO_2 increased the *Deschampsia* root biomass from October (O-horizon to 10 cm depth) by 43% (P=0.06). This is in agreement with other grasslands studies, ranging from 7% increase in root biomass (Jackson and Reynolds, 1996), to 98% increase in net production in elevated CO_2 (in-growth core) (Sindhoj *et al.* 2004) In *Calluna* a clear CO_2 response was only seen in the in-growth cores, and a response in undisturbed areas might become pronounced only after disturbances or after a longer time period.

Besides the increased root growth, elevated CO_2 is expected to increase the water use efficiency and photosynthesis of plants (Rogers *et al.* 1994). In the CLIMAITE experiment, Albert *et al.* (2011a, 2011b) found increased net photosynthesis and increased leaf C/N ratios in response to elevated CO_2. Increased root C/N ratio was also seen in the present study, which supports our hypothesis of improved carbon status of the plant and explains the higher allocation to the roots grown under elevated CO_2. As there is no persistent changes in aboveground biomass change at the site (Kongstad *et al*, 2011), this indicates that the higher root growth in the CO_2 treated plots is a response to limited nitrogen supply, as larger root systems relative to shoots are common when N is more limiting. It is often observed that changes in root-to-shoot ratio in response to CO_2 enrichment depend on soil nutrient availability, i.e. increased root-to-shoot ratio is often associated with nutrient limitation (Poorter and Nagel, 2000).

The root biomass generally did not respond as much to the single treatments of elevated temperature and drought as expected, neither in in-growth cores nor in undisturbed areas.

The few drought effects seen were found mostly in the O-horizon, where the drought effect on soil moisture is also most pronounced. However the combined treatment of drought and elevated temperature seems to be a key interaction for the roots and acted differently between the two dominant species. The root biomass from *Deschampsia* was negatively affected in the TD

treatment, while the opposite was true for *Calluna*. The explanation for this could be the leaf dieback that *Deschampsia* experiences during drought (Kongstad *et al*., 2011). The same adjustment in live biomass has been reported for the roots, and many grass species cope with dry soil by rapidly shedding the fine lateral roots (Eissenstat and Yanai, 1997). The combined treatment of temperature and drought (TD) had the lowest soil water content in the summer of 2007 and 2008 (0-60 cm depth, data not shown), as warming may stimulate evapotranspiration and decrease the soil water content (Bai *et al.* 2010). This probably increased *Deschampsia* fine root mortality during the drought, which gave *Calluna* a competitive advantage where the roots can grow without competition from *Deschampsia* roots, and this drought effect was apparently sustained until autumn/winter. Andresen *et al*. (2010b) also reported increased aboveground *Calluna* growth and reduced *Deschampsia* biomass in response to drought.

In general, the full factor combination mimicking the future climate has been reported to moderate the response of single factor treatments due to antagonistic responses across a large number of ecosystem compartments and processes (Larsen *et al.* 2011). However, the root parameters observed in this study showed a clear effect of CO_2 on root growth, with no significant antagonistic effects of temperature and drought.

Root N concentration and nitrogen uptake

Our results support the hypothesis that N concentration decreases in plants grown under elevated CO_2. The N concentration decreased in elevated CO_2 plots in both *Calluna* and *Deschampsia* roots (mean of all depths) by 8.7 % and 9.1% respectively, similar to Cotrufo *et al.* (1998) who found a mean decrease of 9 % in their synthesis, covering 378 observations. Elevated CO_2 commonly results in lower tissue N concentration, for reasons that are not fully understood but include the dilution effect due to accumulation of non-structural carbohydrates (Bassirirad *et al.* 1997; Taub and Wang, 2008). Such biomass dilution may decrease the concentrations of all soil derived nutrients, while mobile nutrients are additionally decreased due to restricted mass flow (Taub and Wang, 2008). As CO_2 decreases the transpiration, the transpiration-driven mass flow of N is strongly affected.

A decline in tissue nutrient concentration, in addition to uptake and transport capacity of the root, can also be influenced by other processes such as changes in nutrient use efficiency and remobilization, changes in root:shoot ratio and developmental stages of growth (Bassirirad *et al.* 1997; Bassirirad, 2000).

Results from a short term experiment outside the study area indicate that the ecosystem at present is close to N saturation (Larsen *et al.* 2011, Nielsen

et al. 2009). However, roots grown in elevated CO_2 had reduced N concentration which indicates that N is periodically limiting growth, as previously discussed. The root N pool was higher in elevated CO_2 plots and the overall increased uptake of N in response to elevated CO_2 was probably due to increased root growth exploiting a larger soil volume.

Our results from the ^{15}N-assay do not support the hypothesis that nitrogen uptake would increase under elevated CO_2. Hence, roots from CO_2 treated plots did not significantly induce compensatory changes in NH_4^+-N or NO_3^--N uptake in neither species nor any depth. This means that if our results are consistent with no increases in nutrient uptake, other compensatory adjustments by the plants may take place. In the short term increased nutrient-use efficiency can be a solution to decreased nutrient availability, but cannot be sustained indefinitely (see review by Norby and Jackson, 2000). Changes in morphology and life span, along with increased nutrient use efficiency, can prevent the need for a positive adjustment in uptake kinetics (Bielenberg and Bassirirad, 2005). If a greater proportion of the additional carbon resulting from growth under elevated CO_2 is allocated to root growth, then a high CO_2 concentration can lead to an increased N uptake on whole plant basis, although the uptake rate per unit root mass remains unchanged (Bassirirad *et al.* 1999). However, there might be ecosystem-levels constraints to the total size of the root system (Norby and Jackson, 2000). This is not the case in the in-growth cores, but could be a problem in undisturbed soil in the long term. Plants can also increase their potential nutrient uptake rate by producing a greater length of roots from a given mass of roots (i.e. increasing the specific root length, SRL) (Hutchings and John, 2003).

Different studies have provided highly variable results with increasing, decreasing or unchanged uptake under elevated CO_2 (Bassirirad, 2000; Bielenberg and Bassirirad, 2005; Gavito *et al.* 2001; Jackson and Reynolds, 1996; Newbery *et al.* 1995; Taub and Wang, 2008). In the review of Rogers *et al*. (1994) whole plant nutrient uptake was increased for many species under elevated CO_2, but the concentration and nutrient uptake efficiency of most nutrients on a per weight of tissue basis was found to decline. Dijkstra *et al*. (2010) found that increase in plants biomass with CO_2 enrichment was mostly a result of increased nitrogen use efficiency (NUE), while increased plant N uptake contributed to the increase in biomass with increased soil moisture.

Treatment effects were less pronounced than differences between soil layers and differential root uptake capacities in different depths seemed to be more decisive for uptake than the treatment effects. The NH_4^+-N and NO_3^--N uptake did not decrease with depth, as expected, as the availability of NH_4^+-N is most often higher in the top soil were mineralization is largest. This corresponds to Göransson *et al*. (2006) who did not find any significant differ-

ences in uptake rate of NH_4^+-N at different soil depths in a beech and a spruce stand in Denmark.

The largest root biomass and the highest N uptake were found in the same depth of 0-5 cm. The N concentration of the roots decreased with increasing soil depth but no correlation was found between the uptake rate of NH_4^+-N and NO_3^--N and the root nitrogen concentration, as also seen in the study of Goransson *et al.* (2007).

Although *Deschampsia* and *Calluna* acquire organic N forms as amino acids (Andresen *et al.* 2010a), NH_4^+-N and NO_3^--N are probably the most important sources of nitrogen. As assimilation of NO_3^--N in roots have high energy requirements, NH_4^+-N was expected to be the preferred nitrogen form (Marschner, 1995), which was also found in this study. Although the roots of both species and all depths were able to absorb both N-forms, NH_4^+-N was always taken up at a greater rate, on average at a four-fold higher rate. The uptake ratio of NH_4^+-N to NO_3^--N corresponds well with that of inorganic N in the soil, with NH_4^+-N roughly 4 and 10 times higher than NO_3^--N in *Calluna* and *Deschampsia* soil, respectively (Andresen *et al.* 2010a). Our results did not indicate that elevated CO_2 increased the relative preference of NO_3^--N to NH_4^+-N. Others studies also found either no or even negative responses on NO_3^--N uptake rate under elevated CO_2 (Jackson and Reynolds, 1996).

In the combined treatment TD the NO_3^--N uptake was low (although not significantly) in both species and both depths, while the respective nitrogen concentration in TD was not lower than in the other treatments. As low uptake of ^{15}N indicates lack of nutrient limitation in the plant, the explanation for low uptake in the TD treatment could be due to higher nutrient availability, caused by delayed mineralization after an intensive drought during summer combined with lysis of microbial nitrogen. In most plants root kinetics of N uptake is regulated by demand which is likely to exhibit a seasonal pattern (Gessler *et al.* 1998). Seasonal activities of soil organisms produce substantial changes in the availability of required nutrients (Glass, 2005). Therefore this winter study may not fully describe the overall CO_2 effects on N uptake in this mixed heath land / grassland.

The manipulation effects of T and D were mostly moderate and the response to the full treatment combination TDCO2, which mimic the future climate, was not straightforward to interpret from single factor effects. Larsen *et al* (2011) found more antagonistic responses to the treatment interactions, i.e. responses were smaller in combinations than in single treatments.

Results from the first 2 years of treatments at the CLIMAITE experiment indicate that N mineralization will be reduced in the full future climate scenario, and that N leaching is likely to increase (Larsen *et al.* 2011). These changes can in the long term lead to reduced N availability and progressive nitrogen limitation under future climate change. However, although the N

concentration in the roots decreased, the overall root N pool increased due to elevated CO_2. The increased root growth in elevated CO_2 will add more belowground litter to the soil, and C will be accumulated in a pool with relatively slow turnover compared to aboveground parts, at least for the non-woody *Deschampsia*. The root litter has higher C/N ratio and lignin content and a lower turn-over rate with possible positive effect on soil carbon storage.

In conclusion the root growth was stimulated by elevated CO_2 leading to a higher biomass but with lower nitrogen concentration, due to the dilution effect resulting in a higher C/N ratio. However, the overall N content per unit area was increased demonstrating an increased N allocation.

Our results from the in-growth cores, however, suggest that the stimulated root growth does not compensate for the higher plant growth in the CO_2 plots by a corresponding increase in nutrient uptake, as the roots were unsuccessful in converting the additional carbon into increased nitrogen uptake per unit root during winter. The increased root growth in elevated CO_2 plots might be a strategy for the plants to cope with low nutrient supply and leads to an increased N pool size on a whole plant basis.

NH_4^+-N was the preferred nitrogen form, reflecting the differential abundance of the two inorganic N forms. Treatment effects were relatively small relative to differences between soil layers, species and N form.

Warming did not increase root growth and drought had only small negative effects in the organic horizon, while the combination of elevated temperature and drought negatively affected *Deschampsia flexuosa* root biomass, and having the opposite effect on *Calluna vulgaris* roots, probably due to different growth strategies. The full combination of $TDCO_2$ is the treatment that mimics the future climate. In general, the CO_2 response in the measured root parameters was neither significantly moderated by the full factor combination, nor did we observe a synergistic effect of the manipulations, as the effects of T and D were moderate.

Acknowledgements

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TABLE

Table 1. *Fine root plant nitrogen pool (mg N m^{-2}) under Calluna (C) and Deschampsia (D) plants in three soil depths, O-horizon (O-hor), 0-5 cm and 5-10 cm depth. The treatments are: A (ambient), CO$_2$ (elevated CO$_2$), D (drought) and T (elevated temperature) and their treatment combinations TD, DCO2, TCO2 and DTCO2. Values are mean±1SE and significant effects of main treatments are indicated with D, T and CO2 and the interactions indicated as T×D in the last column.*

	A	T	D	TD	CO2	TCO2	DCO2	TDCO2	P value
C O-hor	116±32	156±52	117±39	149±72	181±63	213±50	195±52	202±46	CO$_2$: 0.004
D O-hor	171±67	332±98	138±30	106±29	170±30	314±40	241±65	172±23	T×D: 0.020
C 0-5	538±88	535±95	567±100	834±168	687±27	903±199	685±100	656±106	
D 0-5	474±49	443±67	433±95	451±120	637±75	837±137	636±123	567±57	CO$_2$: 0.051
C 5-10	372±70	381±117	382±104	572±160	392±38	567±152	436±64	387±87	
D 5-10	222±36	256±53	365±76	285±88	352±72	435±946	400±170	328±49	

FIGURE LEGENDS

Fig.1. Average precipitation (mm), air temperature (^oC) and soil water content (vol %) in 2007 and 2008 in control plots (treatment A).

Fig. 2. Total root biomass (g m^{-2}) in October 2008 after 3 years of treatment, in Calluna and Deschampsia (mean±SE). P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig 3. Root biomass (g m^{-2}) from in-growth cores, in Calluna and Deschampsia (mean±SE). P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig 4. Nitrogen concentration (%) in Calluna and Deschampsia roots, in three soil depths, from in-growth cores (mean±SE). P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig. 5. Root NH_4^+-N uptake (ug^{15}N/g DW/2hr) in Calluna and Deschampsia roots in three depths, from the in-growth cores (mean±SE). P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig. 6. Root NO_3^--N uptake (ug^{15}N/g DW/2hr) in Calluna and Deschampsia roots in two depths, from the in-growth cores (mean±SE). There were no data available for the organic horizon.
P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO_2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.
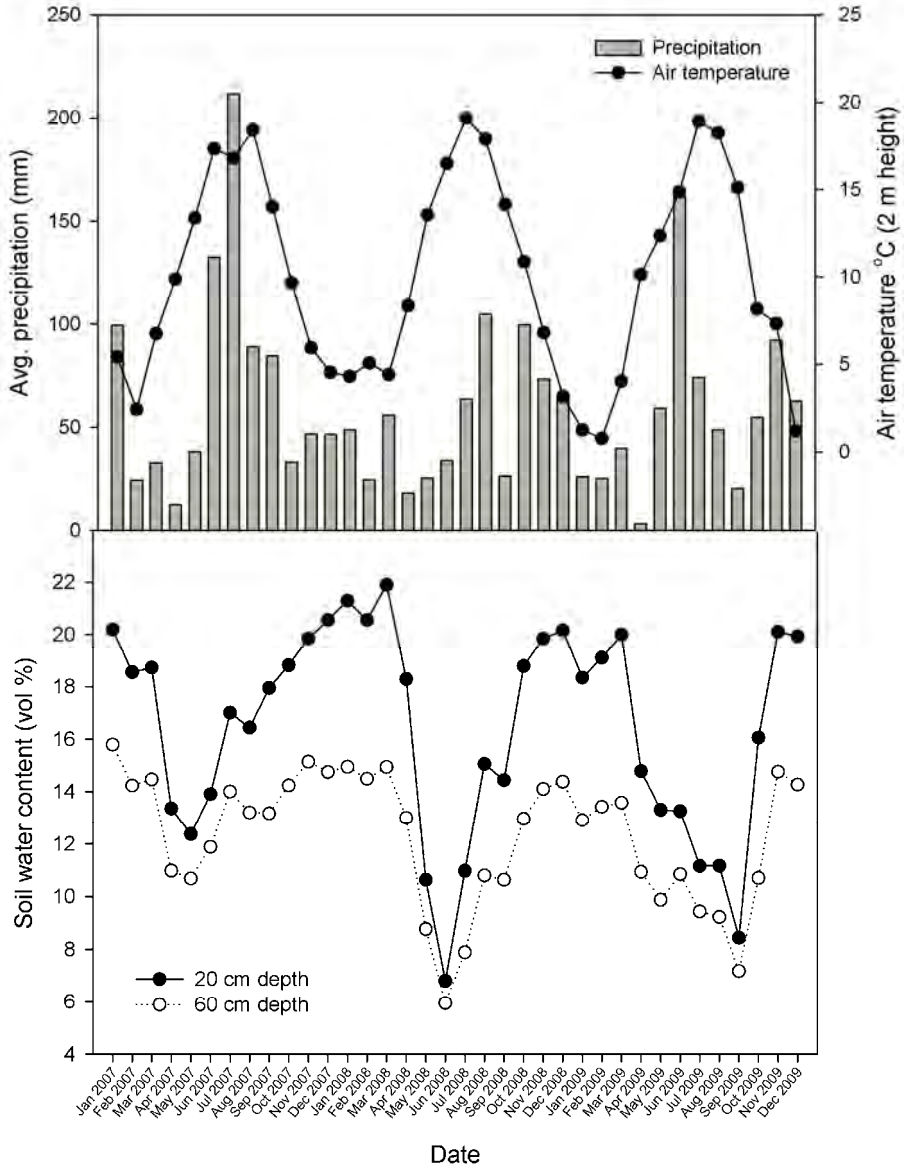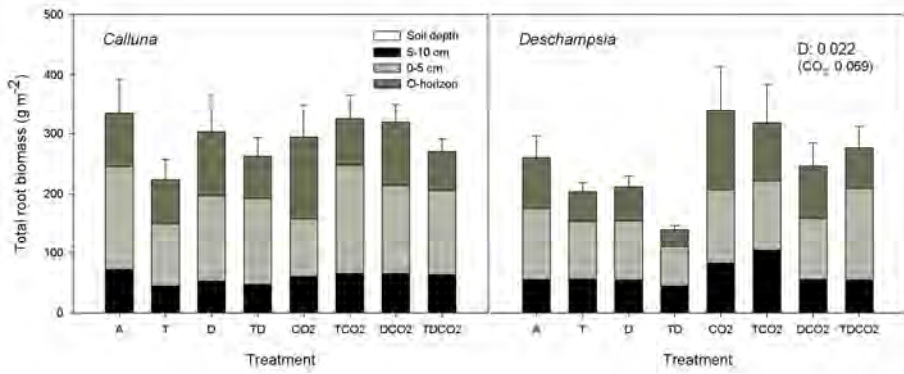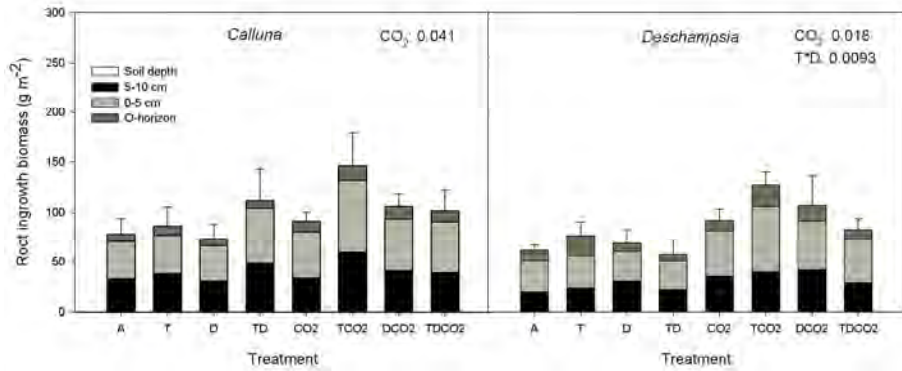
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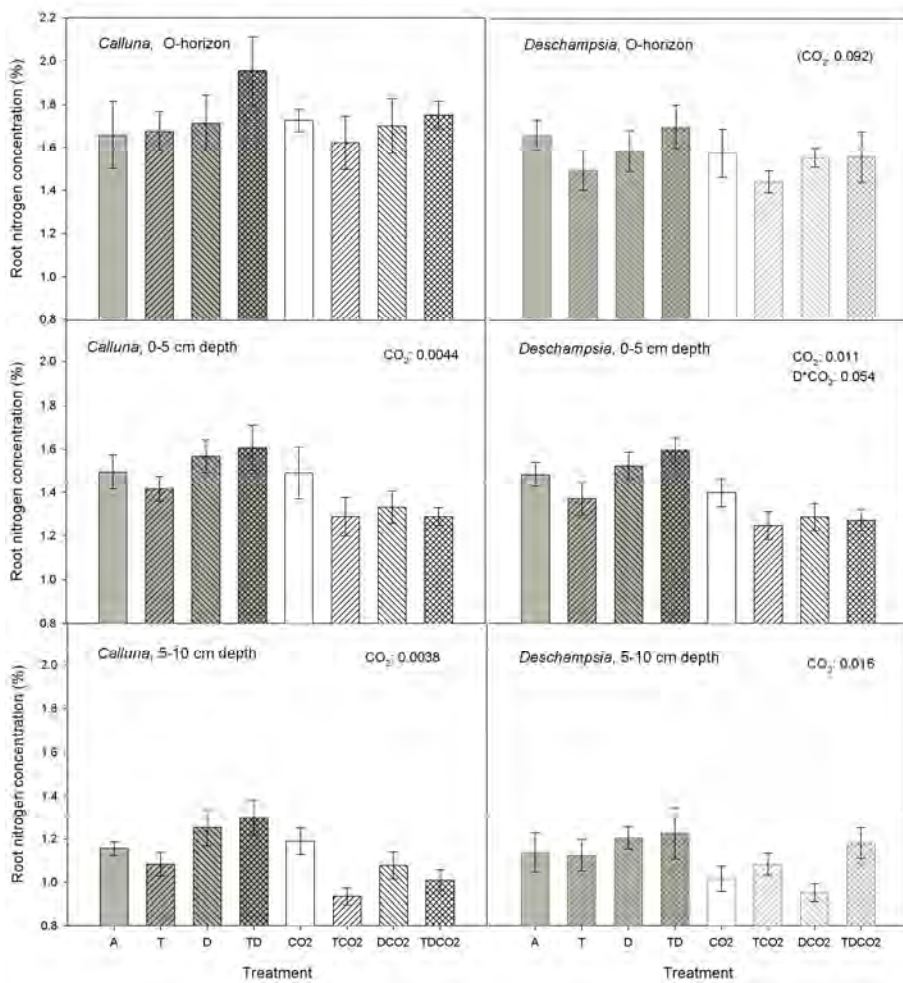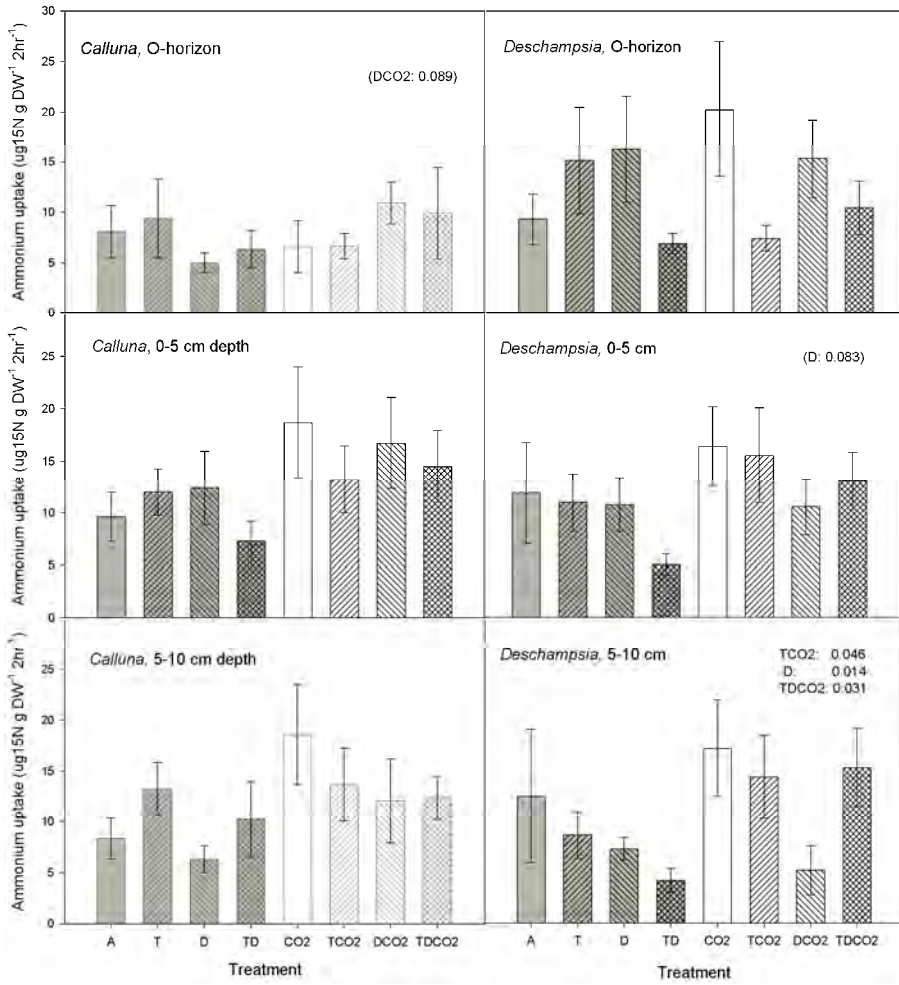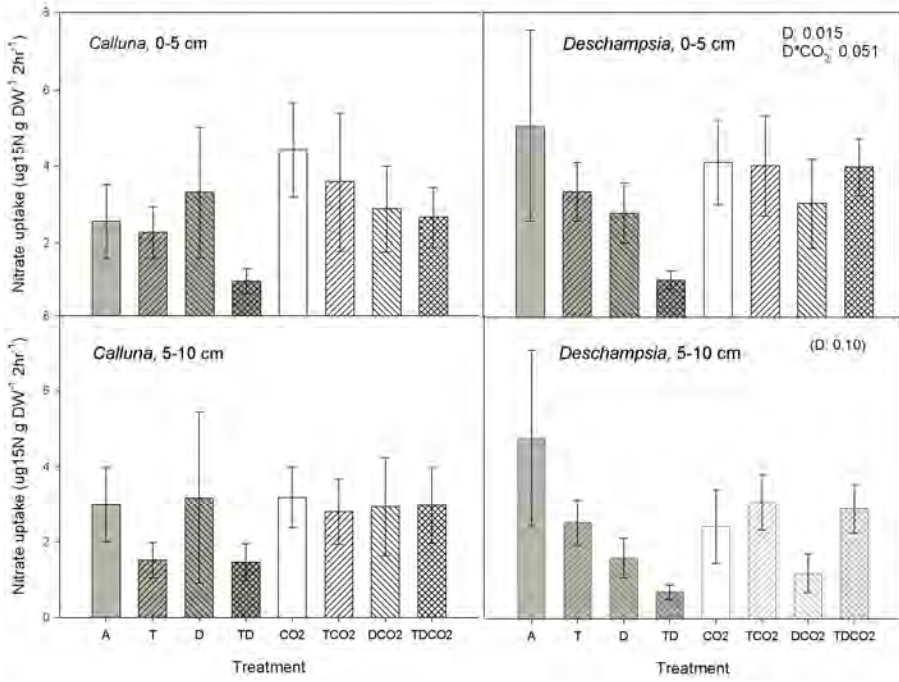

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Fig. 6.

PAPER III

ROOT GROWTH AND NUTRIENT ACQUISITION IN RESPONSE TO FUTURE CLIMATE CHANGE IN TWO HEATHLAND PLANTS

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ABSTRACT

In determining plant responses to a future climate, it is essential to understand how nutrient acquisition of plants and mycorrhizas are affected under climate change as low nutrient availability may constrain plant productivity.

Higher temperature and elevated CO_2 is expected to increase plant C assimilation but it is still not clear how this affect plant nutrient uptake and mycorrhizal colonization of roots. We therefore conducted a field experiment with the aim to investigate nutrient uptake under future climate conditions.

The root growth of the heathland plants exposed to field conditions of a future climate with elevated CO_2, warming and summer drought increased in response to elevated CO_2 for the two dominant species, the grass *Deschampsia flexuosa* and the ericoid dwarf shrub *Calluna vulgaris*. The ericoid mycorrhizal fungi did not respond to the treatments, while the dark septate endophytes of both species were negatively affected by drought. Colonization by arbuscular mycorrhizal fungi increased in response to elevated CO_2.

The two plant species did not increase their uptake of N and P proportionally with root growth, but the root N and P pool were increased under elevated CO_2. In *Calluna* roots a synergistic effect resulted in a higher root biomass N and P pool in the full treatment combination mimicking the future climate in NW Europe. The higher pools of N and P suggest that the plants at present are not strongly nutrient limited when exposed to elevated CO_2 and warming, although decreased root N concentrations in *Deschampsia* might indicate onset of progressive nitrogen limitation during the most intense period of growth. This is supported by the increased uptake of NH_4^+-N per unit root mass. At this time P limitation also seems to be alleviated by

warming in *Calluna* roots, as indicated by lower P demand, probably due to higher mineralization.

The difference in response to the treatments among species suggests different sensitivity to global change factors. This could result in changes in the plant competition as well as changes in belowground nutrient pools in response to future climate change.

INTRODUCTION

Climate change is expected to affect terrestrial ecosystems across the globe with increased atmospheric CO_2 concentration, higher temperatures and changes in the precipitation patterns (IPCC, 2007). Research into climate change effects has focussed much on the aboveground effects, while belowground effects are less studied (Bassirirad *et al.*, 2001; Staddon *et al.*, 2002; Olsrud *et al.*, 2010; Pendall *et al.*, 2008). One important question is how plants acquire growth limiting nutrients in a future climate (Bassirirad, 2000), as low nutrient availability may constrain responses in aboveground plant biomass (White & Hammond, 2008). As a response to nutrient limitation plants can invest a greater proportion of their biomass in the root system to explore a greater soil volume, enhance root physiological uptake capacity, or increase the nutrient-use-efficiency (Bassirirad *et al.*, 2001).

Fine roots and their associated mycorrhizas are the primary pathway for nutrient uptake in most plants. In nutrient limited ecosystems such as heathlands in NW Europe, mycorrhizal symbioses play an important role in nutrient uptake of dominant plant species as *Calluna vulgaris* L. (Hull) and *Deschampsia flexuosa* L. (Trin). *Deschampsia* roots host fungal endophytes, among which the symbiotic relationship with arbuscular mycorhizal (AM) fungi is the best described. AM fungi enhance plant nutrient uptake, especially of phosphorus, and by associating with mycorrhizal fungi the root gains access to P from a larger soil volume (Smith & Read, 2008). AM fungi might also affect water relations (Auge, 2001). Up to 20 % of the net primary production can be allocated to AM fungi (Jakobsen & Rosendahl, 1990); thus AM fungi potentially play an important role for the terrestrial carbon cycle.

Roots of *Calluna* are likely to be colonized by ericoid mycorrhizal (ErM) fungi. ErM fungi are capable of improving host plant nutrient uptake since they can access organic nitrogen by production of extracellular enzymes which release amino acids and –sugars from dead organic matter (Näsholm *et al.*, 1998). ErM fungi have shown the ability to degrade a vast range of recalcitrant compounds and they thus have the potential to affect soil organic matter decay (Read *et al.*, 2004).

Furthermore both *Deschampsia* and *Calluna* may additionally be colonized by dark septate endophytes (DSE) that coexist with mycorrhizal fungi and are conidial or sterile fungal endophytes likely to be ascomycetes. It has been suggested that DSE have the potential to affect plant nutrient uptake and plant responses to pathogens (Mandyam & Jumpponen, 2005). DSE were found to be able to mineralize amino acids in the rhizosphere and make N more available to the roots (Upson *et al.*, 2009).

Elevated CO_2, increased temperature, and water stress are all important factors that may affect the availability and uptake of nutrients in the future. Plant growth has often been shown to increase under elevated CO_2 (Rogers *et al.*, 1994) with more carbon being allocated to roots (Fitter *et al.*, 1997; VanVuuren *et al.*, 1997). Elevated CO_2 has been shown to increase plant root biomass in different kinds of grassland (Adair *et al.*, 2009; Anderson *et al.*, 2010; Dijkstra *et al.*, 2010; Fitter *et al.*, 1997). However, it is still not clear whether plants can exchange additional C for nutrients under climate change (Bassirirad *et al.*, 2001). The plant nutrient uptake under elevated CO_2 will be regulated by compensatory adjustments (Bassirirad, 2000), and one potential mechanism for increasing the nutrient acquisition is stimulation of the mycorrhizal association (Gifford *et al.*, 2000). AM fungi have been found to respond positively to increased atmospheric CO_2 concentrations (Treseder, 2004) and in a subarctic forest ecosystem it has been observed that ErM colonization increased under elevated atmospheric CO_2 concentrations (Olsrud *et al.*, 2010).

Gifford *et al* (2000) report of studies where P concentration of leaves and roots were not affected by elevated CO_2, but most studies show a decline in tissue N and P concentration under elevated CO_2 (Gifford *et al.*, 2000; Taub & Wang, 2008). However, root uptake responses to elevated CO_2 have shown highly inconsistent patterns (Bielenberg & Bassirirad, 2005), due to differences in experimental protocols but also due to species specific responses (Bassirirad, 2000).

Higher temperature is expected to increase length of growing season (Beier, 2004), and increase net mineralization (Rustad *et al.*, 2001; Schmidt *et al.*, 2002) leading to higher nutrient availability, which might increase plant nutrient acquisition. Depending on soil water content, fine root length and mortality (Edwards *et al.*, 2004) also seems to increase with soil temperature (Pregitzer *et al.*, 2000). Warming has been observed to cause positive AM fungal responses (Rillig *et al.*, 2002; Staddon *et al.*, 2004). Only few studies have investigated the effects of global change on DSE, but in a subarctic birch forest understory increased temperature caused an increase in root length colonized by DSE (Olsrud *et al.*, 2010). It is uncertain whether a higher mineralization, mycorrhizal colonization and nutrient availability in a future climate will be sufficient to meet the demand under elevated CO_2 (Bassirirad, 2000). Over longer periods of time nutrient stocks may become

depleted (Pregitzer & King, 2005), leading to progressive nitrogen limitation, where available soil N becomes increasingly limiting as C and N are sequestered in long living biomass and soil organic matter (Luo *et al.*, 2004).

During a drought period where the soil dries, fine roots may desiccate and the nutrient uptake is reduced due to decreased root activity and nutrient mobility (Hinsinger *et al*., 2009). As resources are less available under drought, the competition for limiting nutrients increase in dry soil (Hinsinger *et al*., 2009). Prolonged summer drought has been found to increase the proportion of root length colonized of AM while decreasing the density of external mycelial hyphae (Staddon *et al*., 2003).

Effects of changed climate variables, such as elevated CO_2 or warming have often been studied individually, while the interactions between these factors have been less studied (Beier, 2004), in particular in relation to plant nutrient uptake capacity (Gutschick & Pushnik, 2005). We therefore exposed a Danish heathland to a future climate with elevated atmospheric CO_2 concentration, extended summer drought and higher temperatures in all combinations. In this paper we describe how our future climate change might affect root growth, root N and P and mycorrhizal colonization, after 5 years of treatments. We also investigated the root N and P uptake which reflects the nutrient demand specifically at the time of sampling of roots during the most intensive period of root growth.

We hypothesize increased root growth in elevated CO_2 resulting in higher demand and uptake of N and P, but decreased N and P concentration due to insufficient supply. We also hypothesized that mycorrhizal colonization would be enhanced under elevated CO_2, warming and drought. The nutrient demand and hence uptake was expected to decrease under warming, due to increased mineralization, while drought was expected to increase the root nutrient demand. Drought was expected to slow down root growth, while the temperature treatment might increase root growth, depending on soil moisture content

MATERIALS AND METHODS

Site description

The experimental site is situated in a dry heathland/grass land app. 50 km NW of Copenhagen (55◦ 53' N, 11◦ 58' E), Denmark, on a hilly nutrient-poor acid sandy deposit. The soil consists of 71.5 % sand, 20.5 % coarse sand, 5.8 % silt and 2.2 % clay (Nielsen *et al.*, 2009). The soil is well drained with a pH_{CaCl2} in the topsoil of 3.3 increasing to 4.5 in the B-horizon and an organic top layer of 2-5 cm (O-horizon).

The yearly mean temperature is 8 °C and the yearly mean precipitation is 613 mm (Danish Meteorological Institute, 2009). The site has relatively low atmospheric N bulk deposition of 1.35 ± 0.04 g N m^{-2} y^{-1} in 2007 (Larsen *et al.*, 2011). The experimental area has been managed by sporadically cutting, removal of trees and natural grazing until 2004.

The dominant and studied plant species are the evergreen dwarf shrub *Calluna vulgaris* (c. 30 % cover) and the perennial grass *Deschampsia flexuosa* (c. 70 % cover). Few other grasses, herbs and mosses common for acidic grassland are also present at the site.

Experimental design

The CLIMAITE manipulation experiment started in 2004 with some pre-treatments and in 2005 the climate treatments began. The treatments were made to match the Danish climate scenario in 2075, as predicted by the Danish Meteorological Institute, except that our summer drought is not accompanied by increased winter precipitation.

The experiment consists of 12 octagons (7 m in diameter) laid out pair wise in 6 blocks. Each block consists of two octagons with one octagon receiving elevated CO_2 by FACE technique and the other receiving ambient CO_2 (A). Within each octagon there are four subplots with the following treatments: summer drought (D, exclusion of rain by automatic shelters), elevated temperature (T, passive night time warming by reflective curtains), a combination of drought and elevated temperature (TD), and an untreated control for reference (A). The experiment provides a full factorial design replicated 6 times with the treatments and combinations: A, T, D, CO2, TD, TCO2, DCO2 and TDCO2 giving a total of 48 plots.

The elevated CO_2 plots are enriched with CO_2 by FACE (Free Air Carbon Enrichment) technique and have a target of 510 ppm. The enrichment starts at dawn and continues until dusk. During night and with complete snow cover the CO_2 is turned off. The temperature treatment (T) elevates the temperature by 1-2 °C by passive night time warming with reflectance curtains from dusk till dawn. Drought (D) is exposed to the site once or twice every year in spring or summer time by exclusion of rain by automatic shelters. The drought continues for 2-5 weeks or until soil water content gets below 5 vol % water content in the top 20 cm of the soil. See Mikkelsen *et al*. (2008) and Larsen *et al*. (2011) for further details on experimental design and set up. The drought period in 2010, was from May 4 to June 3 and excluded 70 mm of rain. Figure 1 shows the climatic data at the site and soil moisture conditions in control plots from 2009-2010.

ROOT GROWTH

Relative root growth was estimated by in-growth cores. In each experimental plot, a soil core was sampled under *Calluna* and *Deschampsia*, respectively. The soil was sorted free of roots and was put into mesh bags with a diameter of 5 cm and a mesh size of 1 mm. The soil was placed in the same original order of depth, divided into O-horizon, 0-5 cm and 5-10 cm depth, separated from each other by a small cloth inside the bag. The in-growth cores were installed in the soil in January 2009, and taken up in June 2010 by the use of a soil auger slightly bigger than the diameter of the in-growth bags. The 96 samples were brought back to the lab and roots were separated from the soil by use of sieves and hand picking of roots with forceps, then washed carefully, and used for the ^{15}N and ^{32}P assay. Later we estimated the biomass dry weight (g m^{-2}) of the three separate soil depths and two dominant species.

NITROGEN UPTAKE AND CONCENTRATION

The study of nitrogen uptake was done by ^{15}N assay (Jones *et al*. 1991), and used roots from the in-growth cores. All roots had a maximum age of 18 months. Due to the large number of samples and the time demanding root sorting procedure, it was only possible to harvest and analyze 3 blocks (6 octagons) at a time. Hence, the first three blocks were harvested the 21st of June and the next three blocks the 26th of June 2010. Soil samples were kept cool until the roots were carefully removed from the soil in the lab. The roots were not separated into species, but we assumed that the majority of the roots would belong to the species under which the in-growth bags were placed. However, there was some growth of grass roots in the *Calluna* samples, while no in-growth of *Calluna* roots were seen in *Deschampsia* samples. The soil samples were separated into O-horizon, 0-5 cm and 5-10 cm, but the O-horizon was not included in the assay due to low biomass. Each horizon was sorted by hand and fine roots were washed in demineralised water. The roots were put in a plastic bag with a damp/moist hand towel cloth and kept dark at 5 °C until the assay was conducted (after max 2 days).

The N bioassay was performed using the method described by Jones *et al*. (1991). All roots of both *Deschampsia* and *Calluna* were approximately of same diameter (< 1 mm in diameter) and were not differentiated into different size classes. All roots were considered active and able to take up nutrients. The fresh root bundles were marked with a name tag and put in a 5 ×10^{-4} M CaCl$_2$ pre-soak solution for 30 minutes. The roots were immersed in the presoak solution to maintain root cell membrane integrity and to remove ammonium, nitrate and nitrite ions from the cell-free space (Rosengren *et al*. 2003). The roots where then transferred to the uptake solution which consisted of 5 ×10^{-4} M CaCl$_2$ labelled with 2 ml ^{15}N-NH$_4$Cl solution and 8 ml of ^{14}N-NH$_4$Cl solution, to get a 20% enrichment of ^{15}N. The uptake ^{15}N

solutions were made of 0.0728 g 100% ^{15}N-NH$_4$Cl into 200 ml liquids. The roots were in the ^{15}N-solutions for 2 hours, and then washed in running de-mineralised water for 15 minutes, put in paper bags and dried in the oven at 80 °C. Finally the roots were ground and weighed and ^{15}N, % N and % C were analyzed with a Eurovector elemental analyzer (Milan, Italy) coupled to an Isoprime mass spectrometer (Cheadle Hulme, UK). Excess ^{15}N (atom %) was converted to absorption rate of N (in µg N/ g root DW / 2 h). The total N concentration was also calculated, with subtraction of N that had been taken up during the bioassay (Michelsen *et al.* 1999). High ^{15}N uptake demonstrates nitrogen limitation (Jones *et al.*, 1991; Rosengren *et al.*, 2003).

The relative standing pool of belowground plant nitrogen was calculated as the root biomass from the in-growth cores multiplied by the nitrogen concentrations.

PHOSPHORUS UPTAKE AND CONCENTRATION

The P assay was conducted according to Harrison and Helliwell (1979) and Michelsen *et al.* (1999). Live roots were separated and washed as described above, marked with a nametag and put in a pre-soak uptake solution of 5 $\times 10^{-4}$ M CaSO$_4$,2H$_2$O for 30 minutes. The presoak solution was used in order to maintain cell membrane integrity and to leach out any physically absorbed P from root free space. The roots were then transferred to an uptake solution of 5 $\times 10^{-4}$ M Calcium sulphate x 5 $\times 10^{-6}$ M KH$_2$PO$_4$, and 2.0 MBq ^{32}P l^{-1} as orthophosphate for 15 min.

Unabsorbed ^{32}P where washed from the roots in running de-ionized water for 5 min. Roots were then transferred to 20 ml scintillation vials with 15 ml H$_2$O and the Cerenkov radiation was quantified in a Packard 1900TR liquid scintillation counter (Packard Instrument Co., Meriden, CT, USA). Each root sample was then removed from its vial and the ^{32}P remaining in the vial was recounted under identical conditions. This second count represented the ^{32}P that was not metabolically absorbed by the root and was subtracted from the first count. Root absorption of ^{32}P was calculated as pg P mg^{-1} fresh weight of root 15 min^{-1}.

The root samples were blotted on paper towel, stored in 50 % ethanol and later analysed for mycorrhizal colonization after isotope decay.

P concentration was analyzed on a subsample, with flow injection after acid digestion.

DETERMINATION OF MYCORRHIZAL COLONIZATION

After isotope decay roots were visually examined to determine plant species and fungal colonization. The roots were cut into pieces of ca. 1 cm length and cleared by leaving samples in 10 % KOH for 14 h at room temperature. Subsequently roots were washed and stained with a 5 % ink-vinegar solution

(Vierheilig *et al.*, 1998) and destained in lactoglycerol. This was followed by visual examination according to the magnified intersections method as described by McGonigle *et al.* (1990. For each sample one slide was made with root pieces of ca. 1 cm length aligned in 6 rows. With 0.5 cm intervals passes across the slide were made. Every intersection of the vertical eyepiece crosshair was inspected using 400 x magnifications. On average 45 intersections were registered per sample.

Roots from *Deschampsia* were distinguished from *Calluna* roots by colour. *Deschampsia* roots were stained blue while *Calluna* roots were light brownish. Intersections were not registered when cortical cells were missing or when species could not be determined. For each intersection the plane focus was moved completely through the sample. The identity of the slides was unknown to the observer.

In *Calluna* roots it was registered if the vertical eye piece hair crossed ErM hyphal coils or DSE hyphae. DSE hyphae were recognized as thick, melanised hyphae with septa.

In *Deschampsia* roots the presence of fine endophyte (FE) colonization, AM colonization and DSE hyphal colonization was registered. Blue coloured hyphae < 2 µm diameter were classified as FE, whereas blue coloured hyphae > 2 µm were classified ad AM. Vesicles were also registered as FE or AM colonization if attached to FE or AM hyphae, respectively. DSE hyphal colonization was examined as described above.

Observations for each sample were based on registration of *Deschampsia* and *Calluna* in the two subsamples. The percentage root length colonized was calculated as the average number of intersections with colonization in both subsamples.

Statistical analysis

Statistical analyses were conducted by using the PROC MIXED procedure of SAS (SAS Institute, 2003). The statistical model chosen included a random statement that accounted for the experimental design (*Random* block octagon octagon×D octagon×T). The same model was used for all variables, with all main factor effects and their interactions.

Data were transformed in case requirements for normality and homogeneity of variances were not met. P-values <0.05 were considered significant, but effects with P<0.1 are also reported. Model reduction was done to obtain the best model by sequential removal of the terms in the model with the highest P value. Differences of Least Squares Means (DLSM) were used to interpret significant interactions.

RESULTS

ROOT BIOMASS

The root biomass sampled from in-growth cores in June is a measure of relative root growth during 18 months. The root biomass in the in-growth cores under the two species was approximately the same (Fig.2), and compared to the initial standing root biomass in the same volume of soil, the root biomass in in-growth cores were 3-4 times smaller. The root in-growth cores were taken up 19 days after the drought treatment ended in June 2010, but there was no effect of drought on root biomass. Root biomass was significantly higher in elevated CO_2 for both species from O-horizon to 10 cm depth. In *Calluna*, there was also a temperature effect, as warming further increased the root biomass.

NITROGEN CONCENTRATION AND N POOL SIZE ESTIMATION

In *Deschampsia* roots the nitrogen concentration was lower in elevated CO_2 in both 0-5 and 5-10 cm depth (Table 1). The nitrogen concentration in *Calluna* roots increased significantly in the drought treatments in 0-5 cm and tended to increase in 5-10 cm depth.

As a reflection of the N concentration, the C:N ratio of *Calluna* roots was negatively affected by drought in 0-5 and tended to be so in 5-10 cm depth. In *Deschampsia* the root C:N ratio increased under elevated CO_2, significantly in 0-5 cm and with a tendency towards this in 5-10 cm depth.

The standing pool of belowground plant nitrogen (calculated from the in-growth root biomass multiplied with the N concentration) showed an increase under elevated CO_2 in both species and in both depths (0-10 cm) (Table 1). Besides the CO_2 effect, warming also increased the N pool and drought tended to do so in *Calluna* in both soil depths. In *Deschampsia* there was also a weak interaction of D×CO_2 in 5-10 cm depth.

AMMONIUM UPTAKE

The nitrogen deficiency test was developed as a tool to relate root nitrogen levels to the fertilizer regime in plantations (Jones *et al.* 1991). The results obtained with this method with excised roots represent a relative measurement of the root net uptake capacity at different soil depths at a particular time point, and not the actual uptake rate as would have been found with the roots still attached to the plant (Goransson *et al.* 2007).

The uptake of NH_4^+-N by excised roots was not significantly affected by treatment in either soil depth in *Calluna* (Fig 3). The results from soil depth 5-10 cm are not shown due to lack of treatment effects. In *Deschampsia* roots an interaction of T×CO2 in 0-5 cm depth was due to the treatments CO2 and DCO2 being higher than all other treatments. This indicates that

CO_2 increased the uptake, while warming reduced the positive effect of elevated CO_2. The uptake in 0-5 cm depth also tended to be positively affected by elevated CO_2. The NH_4^+-N uptake was similar in both *Deschampsia* and *Calluna* roots in both soil depths.

PHOSPHORUS UPTAKE

The ^{32}P uptake by excised roots in *Calluna* in 0-5 cm was significantly reduced by warming (Fig 4) and the uptake was significantly higher in both D and CO_2, compared to the combination with elevated temperature (TD and TCO2) indicating a clear 3-way interaction. The samples from 5-10 cm depths were not sufficiently well replicated, and results are therefore not presented.

In *Deschampsia* roots, there was only a tendency towards a positive drought effect in 0-5 cm depth, leading to higher uptake, while there were no significant effects in 5-10 cm depth.

PHOSPHORUS CONCENTRATION AND P POOL SIZE ESTIMATION

There were no significant treatment effects on root P concentration (Table 2), but there was a tendency in Calluna for a positive effect of drought (P=0.0607). Also an interaction resulted in the P concentration being higher in the single treatment of TD in 0-5 cm depth (T×D×CO2, P=0.0659).

The standing pool of belowground plant phosphorus showed an increase in both species and soil depths under elevated CO_2 and under warming in *Calluna* in both soil depths (Table 2). In *Deschampsia* there was an interaction of T×CO2 in 0-5 cm depth, as the P pool increased even further when the elevated CO_2 treatments were combined with warming.

MYCORRHIZAL COLONIZATION

The percentage root length colonized by AM fungi was stimulated by elevated CO_2 in *Deschampsia* roots in 0-5 cm, and with a tendency towards this in 5-10 cm (Fig. 5a and b). In 5-10 cm there was also a 3-way interaction of T×D×CO2. Evaluation of DLSM showed that drought treatment alone was significantly lower than ambient, DCO_2 and TCO_2 treatments (Fig 5b).

The colonization by dark septate endophytes in *Deschampsia* 0-5 cm was lower than by AM fungi and responded less clearly to the treatments, with many interactions (Fig. 5c and d). The interaction of T×D in 0-5 cm was, by evaluation of DLSM, explained by a higher colonization when drought and DCO2 were combined with warming. There was also a 3-way interaction, where the DCO2 treatment was significantly lower than CO2 and TDCO2.

In 5-10 cm the interaction of D×CO2 is explained by a lower root colonization of DSE in the drought treatment (D) and drought in combination with temperature (TD).

The fine endophytes of *Deschampsia* did not respond to any treatments in neither soil depth (data not shown).

The ErM fungi did not respond to the treatments in 0-5 cm in, but in 5-10 cm depth there was a significant 3-way interaction (T×D×CO2, P=0.0493) which can be explained by a lower colonization in elevated temperature, as the temperature treatment alone was significantly lower than when combined with drought or elevated CO_2 (data not shown).

The dark septate endophytes in *Calluna* did not respond to any treatments in 0-5 cm depth, but in 5-10 cm depth there was a significant 3-way interaction, caused by a significantly lower colonization in the single effect of drought, compared to A, TD and TCO2 (Fig 5e). These interactions generally indicate a lower colonization in the drought treatments, either drought alone or in combination, except for ErM.

DISCUSSION

After 1.5 years of root growth in in-growth cores, significant effects of both CO_2, warming and drought were seen in this heathland ecosystem. Further the two studied plant species responded differently to the treatments in most of the measured variables, with many interactions. Root growth in in-growth cores of *Calluna* was higher when two or three treatments were combined, compared to the single treatments. This synergistic response after 5 years of treatment is in contrast to an earlier synthesis by Larsen *et al* (2011), in which most treatment effects were antagonistic when measured 2 years after the treatments began. This is important when up scaling from single to multiple factors in this species, and confirms the need for multifactorial experiments with changes in the microclimate of plants.

Biomass

The root growth was increased under elevated CO_2, by 50 % and 57% in *Calluna* and *Deschampsia*, respectively. This corresponds to the response measured by minirhizotrons at the site, where elevated CO_2 increased the root length of mixed vegetation by 45 % (Arndal *et al*, in prep). We expected a high response to elevated CO_2 and other grasslands studies also report of root biomass increases from in-growth cores (Anderson *et al.*, 2010; Milchunas *et al.*, 2005; Sindhoj *et al.*, 2004), although lack of responses has been reported too (Handa *et al.*, 2008).

In consistence with the greater allocation to roots in elevated CO_2, increased photosynthesis and leaf C:N ratios have been observed in both species in the site (Albert *et al.*, 2011b; Albert *et al.*, 2011a), while no persistent effect on aboveground plants biomass have been found (Kongstad *et al.*, 2011). This indicates that beside aboveground increased allocation to reproductive structure (J. Kongstad, pers comm.) the extra C in elevated CO_2 is preferentially allocated belowground, and used for root production, root exudates or mycorrhizal associations.

Calluna root biomass was also positively affected by warming, resulting in a 71 % higher biomass compared to roots not exposed to warming. The increased root biomass may be a result of increased belowground carbon allocation as a result of the observed increase in photosynthesis in elevated temperature, at the site (Albert *et al.*, 2011a). As reviewed by Pregitzer *et al.* (2000) there is evidence that root length extension is positively related to soil temperature. However, increased root accumulation (measured by minirhizotrons) in summer was reported to be more a function of longer growing season than of soil temperature (Fitter *et al.*, 1998). At our experimental site, earlier aboveground growth in spring (Kongstad *et al.*, 2011) may provide the evergreen shrub with a competitive advantage in nutrient uptake compared to the grasses. The temperature treatment in our experimental site increased gross mineralization after the first 2 years of treatment (Larsen *et al* 2011), and as increased soil N availability can increase the rate of root length extension (Pregitzer *et al.*, 2000 and references therein) this might also explain the higher root growth, under the assumption that increased root length equals increase in root biomass.

Nutrient uptake and mycorrhizal colonization

The higher root growth in *Deschampsia* under elevated CO_2 resulted in a lower root N concentration and higher C:N ratio. This was probably due to a N dilution effect, where the increased root growth reduced the root nitrogen concentration due to higher increase in photosynthetic assimilation of carbon relative to plant N uptake. Decreased N concentration in roots in response to elevated CO_2 has been reported by others (Cotrufo *et al.*, 1998; Fitter *et al.*, 1997; Taub & Wang, 2008).

In *Calluna* the higher root N concentration in the drought treated plots resulted in a decreased root C:N ratio, which could be due to less C allocation belowground and lower root growth.

As there were no treatment effects in the ammonium uptake in *Calluna*, the roots did not change the uptake per unit root in concert with the higher growth in elevated CO_2 and warming. Even when supplies of nutrients are freely available, roots do not always take up nutrients proportionally to their growth, which suggest that nutrient supply is not the only factor determining mineral uptake (Newbery *et al.*, 1995). In Scotland *Calluna vulgaris* also did not increase the nutrient uptake with increased growth under elevated CO_2, suggesting that the growth response to elevated CO_2 would be limited by nutrient availability (Woodin *et al.*, 1992).

There was a strong negative effect of temperature on the P uptake in *Calluna*, indicating less P demand. In a mediterranean study, warming increased the activity of soil acid phosphatases, leading to a higher P content in plants (Sardans *et al.*, 2006), and probably a lower P demand in the summer. The low uptake in our study suggests that lack of P was not limiting *Calluna* in elevated temperature at this time. Although the root growth was high in warming, it did not lead to any decreases in the P concentration. Nielsen at al. (2009) showed at the same heathland, that N seemed to limit *Calluna* periodically while P seemed to limit *Deschampsia* more (Nielsen *et al.*, 2009). The lower P demand in *Calluna* roots under warming could also be due to plant physiological reasons, as these roots started their growth earlier in the season and therefore had fulfilled their nutrient demand at an earlier stage. However, the lack of treatment effect in the root P concentration of both species, probably reflects a very stable P concentration in the roots, thus P is not diluted to the same extent as nitrogen in response to higher root growth. Few data exist on plant P concentration in response to elevated CO_2 (Gifford *et al.*, 2000), and Gifford concludes that the processes governing plant C:P ratios are probably independent of those determining plant C:N ratios under elevated CO_2. The lack of a clear CO_2 response on root P concentration was also reported in 10 herbaceous species (Staddon *et al.*, 1999).

The P uptake was increased in drought in *Deschampsia*, probably due higher demand after the drought ended, and nutrients were needed to support new leaf production. The uptake was not affected by elevated CO_2, but the higher arbuscular mycorrhizal colonization might be a plant strategy for increasing the P uptake, instead of increasing the plant uptake per unit root. This is described by Newbery *et al*. (1995), who concluded that the grass uptake of N and P was not enhanced proportionally with dry mass under elevated CO_2, even under adequate nutrient supplies. Elevated CO_2 is shown to increase total plant P but only as a result of larger plants (Gavito *et al.*, 2002; Gavito *et al.*, 2003; Rouhier & Read, 1998; Staddon *et al.*, 1998; Staddon *et al.*, 1999). Lack of increased nutrient uptake of N and P despite higher growth in elevated CO_2 has been reported in other studies (see Newbery *et al.*, 1995).

In *Deschampsia*, the most dominant treatment effect was elevated CO_2, which increased the root growth, followed by lower N concentration, higher C:N ratios and a tendency for a higher NH_4^+-N uptake in 0-5 cm. Also the arbuscular mycorrhiza responded by an increase in % colonization under elevated CO_2, as we expected. As reviewed by Treseder (2004) elevated CO_2 increase mycorrhizal colonization, which is likely due to a higher belowground transport of C (Olsrud *et al.*, 2004). However, the nutrient uptake from external hyphae does not necessarily increase the whole plants nutrient uptake (Bassirirad *et al.*, 2001), and our data indicate that the grass roots promote the nutrient uptake under elevated CO_2 by both increasing arbuscular mycorrhizal colonization, root growth and uptake of NH_4^+-N per unit root. AM might also contribute to N uptake, although to a less extent than P uptake (Smith & Smith, 2011). We also saw that the 3-way interaction for AM fungal colonization in 5-10 cm depth could be explained by a negative drought effect. This corresponds with the results presented by Staddon *et al*. (2003). The drought stressed plants may provide less carbon to their AM fungal symbionts thus causing lower colonization. Furthermore this may relate to the increased P uptake seen in the drought treatment, so if the AM symbiosis is less pronounced under drought the plants will be more dependent on their root P uptake.

No treatment effects were seen on the percentage root length colonized by fine endophytes. However, this may be due to difficulty of recognizing fine endophytes when they occur together with the more coarse hyphae of AM fungi.

For *Deschampsia*, warming promoted DSE fungal colonization in the upper most part of the soil, where the warming treatment is also most effective. This response to warming has also been reported for subarctic birch forest understory by Olsrud *et al*. (2010) in a subarctic system. The DSE colonization in both *Calluna* and *Deschampsia* in 5-10 cm depth seems to be lower when drought is applied alone, and could be a negative effect on the low soil

moisture. In an arid ecosystem in New Mexico, DSE colonization of grass roots was more extensive than colonization by AM fungi (Barrow, 2003). There were no correlation between seasonal variation of DSE fungi and precipitation patterns in a prairie ecosystem (Mandyam and Jumpponen (2005). This is in contrast to our results which show that the DSE colonization in both *Calluna* and *Deschampsia* in 5-10 cm depth seems to be lower when drought is applied alone. So even though DSE fungi are found worldwide and in most ecosystems (Jumpponen & Trappe, 1998) their tolerance to a stress factor as drought varies between ecosystems.

There were some similarities in the responses of DSE colonization from *Calluna* and *Deschampsia* roots. This is in accordance with the apparent lack of host specificity with DSE fungal isolates from ericaceous plants in a Dutch heathland, being able to colonize and increase plant N uptake of both grass root and vice versa (Zijlstra *et al.*, 2005). Also no indications for fungal host preferences were reported in a study of fungal communities on ericaceous roots in Arctic tundra (Walker *et al.*, 2011).

We did not find any indications of higher ErM colonization in elevated CO_2 in our study. ErM have been shown to facilitate decomposition of recalcitrant carbon compounds, and when the allocation of photosynthates to mycorrhizal roots is high, fungi decompose soil organic matter (Bajwa & Read, 1985; Read & Perez-Moreno, 2003; Talbot *et al.*, 2008). In subarctic heath, elevated CO_2 increased the ErM colonization (Olsrud *et al.*, 2010), but the Arctic system is more nutrient limited than our heathland, which might explain the differences in responses. This is also supported by the NH_4^+-N uptake which was also not increased under elevated CO_2, as would probably be the case if the plants were strongly nutrient limited.

Nutrient pools and future climate

The two studied species increased the total root N pool from the in-growth cores in response to elevated CO_2, and in spite of lower N concentrations in Deschampsia roots. In *Calluna* warming and drought also tended to increase the N pool. This synergistic effect resulted in a higher N pool in the full treatment combination of TDCO2, mimicking our future climate which might have implications for C, N and P pools belowground.

As we did not find any indication of a higher ammonium uptake per unit root in *Calluna*, and only a tendency in *Deschampsia*, this suggest that the greater root biomass is responsible for the overall higher plant root N uptake, leading to a higher plant N pool in elevated CO_2 in both species. A combination of increasing fine root production, increased rates of soil organic matter decomposition, and increased allocation of C to mycorrhizal fungi is likely

to account for greater N uptake under elevated CO_2 (Finzi *et al.*, 2007). Their analysis showed that regardless of the specific mechanism, larger quantities of C entering the belowground system under elevated CO_2 results in greater plant N uptake, even in N-limited ecosystems (Finzi *et al.*, 2007). This would result in more N being bound in an organic pool with low turnover, due to recalcitrant components, especially in *Calluna* roots with high lignin content. If more N is stored in an inorganic pool in a future climate, *Calluna* will probably benefit more than *Deschampsia*, as ErM have the potential to affect soil organic matter decay (Read *et al.*, 2004) and can access organic nitrogen by production of extracellular enzymes (Näsholm *et al.*, 1998). However if the nutrient turnover increases in a future climate, with more plant available N, *Deschampsia* will probably have an advantage over *Calluna*. In studies of NE heathlands *Deschampsia* is expected to gain competitive advantages, due to enhanced N deposition (Aerts & Heil, 1993).

We found increased P pools in the warming treatment of *Calluna*, and in the 0-5 cm in *Deschampsia*. This corresponds to Andresen *et al.*, (2010), who also found increased *Calluna* plant N and P pools in response to warming, two years after the treatments began. They suggested that warming alleviate nutrient limitation in *Calluna*, which is also supported by our results with lower P demand in *Calluna* roots in response to warming.
The larger P pool in *Deschampsia* in the CO_2 treatment is also in correspondence with the positive arbuscular mycorrhizal response to elevated CO_2.

We found higher root biomass in response to elevated CO_2 in the two plant species studied, but it is not yet possible to conclude how this will affect the belowground total C and nutrient pools and soil C sequestration. Rhizodeposition, i.e the soil C derived from root turnover, root hair and mycorrhizas, is expected to increase if more C is allocated belowground under elevated CO_2 (Pendall *et al.*, 2004). However the longer term responses might be different from the short term responses and a new steady state is expected, where the extra growth might be nutrient limited, as the plant nutrient uptake does not increase in concert with elevated CO_2. However at present, the higher plant N and P pools suggest that the plants are not strongly nutrient limited when exposed to elevated CO_2 and warming.

CONCLUSION

Elevated CO_2 increased the root growth for both dominant plant species in the temperate heathland, and for *Calluna* also warming had a positive effect on root growth. The effect was synergistic in *Calluna* roots and resulted in a higher plant N and P pool in the full treatment combination of TDCO2, mimicking the future climate in NW Europe.

The grass also had higher N and P pool under elevated CO_2, possibly improving nutrient uptake under elevated CO_2 by increasing arbuscular mycorrhizal colonization, increasing root growth and the uptake of NH_4^+-N per unit root.

Belowground plant N and P pool increased in response to warming and elevated CO_2, indicating that the plants were not strongly nutrient limited.

However in Deschampsia the root N concentration was lower in elevated CO_2, indicating that the nutrient uptake does not fully increase in concert with elevated CO_2 which might result in progressive nitrogen limitation.

The studied plant species and their associated fungal colonizers responded differently to the treatments suggesting different sensitivity to global change factors. This could result in changes in plant competitive interactions as well a belowground nutrient pools in response to future climate change.

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FIGURE LEGENDS

Fig 1. Average precipitation (mm), air temperature (^{o}C) and soil water content (vol %) in 2007-2010 in control plots (treatment A).

Fig 2. Root biomass from in-growth cores in Calluna and Deschampsia (mean±SE). Black part of bars represents 5-10 cm depth, light gray 0-5 cm depth and dark gray is the organic horizon. P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig 3. N bioassay uptake of excised roots of Calluna and Deschampsia (µg ^{15}N/g root DW/2 hr)(mean±1SE) from 0-5 cm depth. No effects were seen in depth 5-10 cm in neither species (not shown). P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig 4. P uptake of excised roots of Calluna and Deschamspsia (pg P/mg root fresh weight/15 min) (mean±1SE). No data available for 5-10 cm depth in neither species. P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations.

Fig 5. Root length colonization (%) of Arbuscular Mycorrhiza (AM) in Deschampsia (a,b) and Dark Septate Endophytes (DSE) in Deschampsia roots in 0-5 and 5-10 cm (c,d), and Dark Septate Endophytes in Calluna roots 5-10 cm (e). P values of treatment effects are shown in the graph. The treatments are: A (ambient), CO2 (elevated CO_2), D (drought) and T (elevated temperature) and all treatment combinations. All values are mean±1SE.
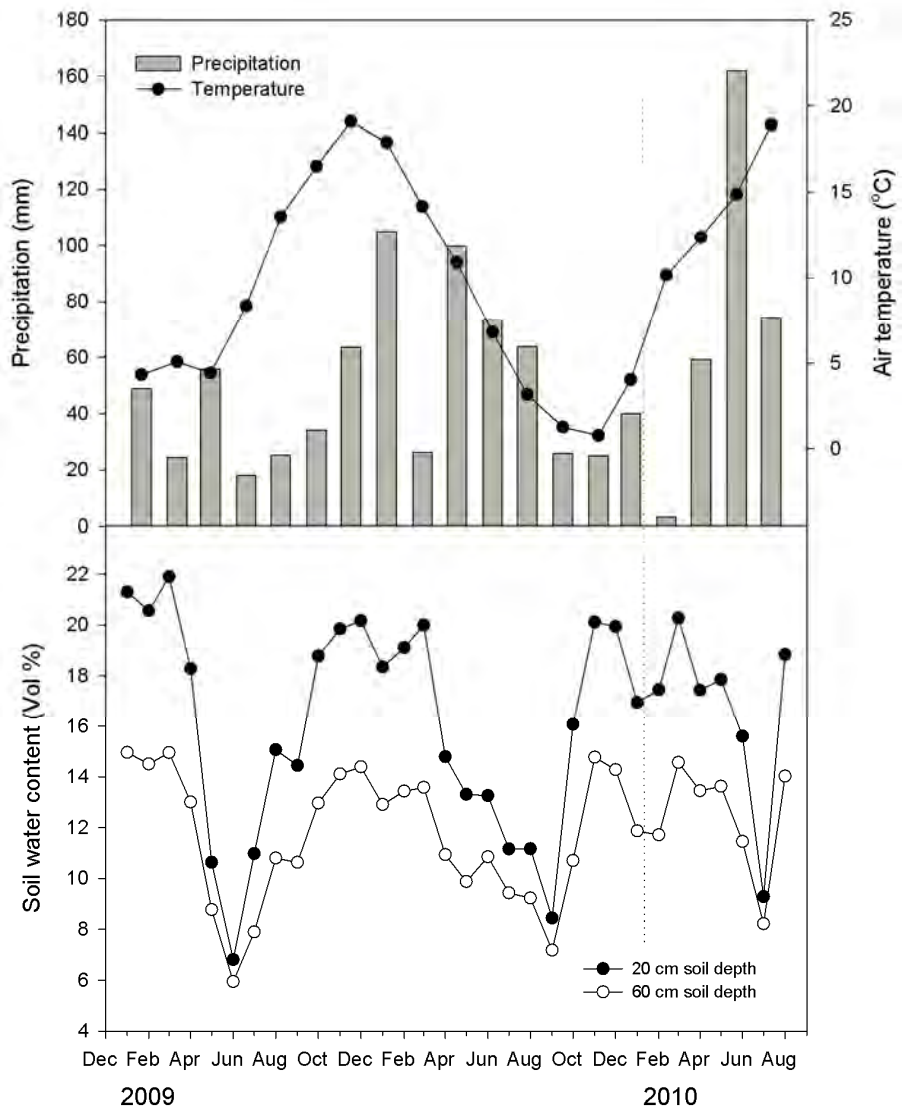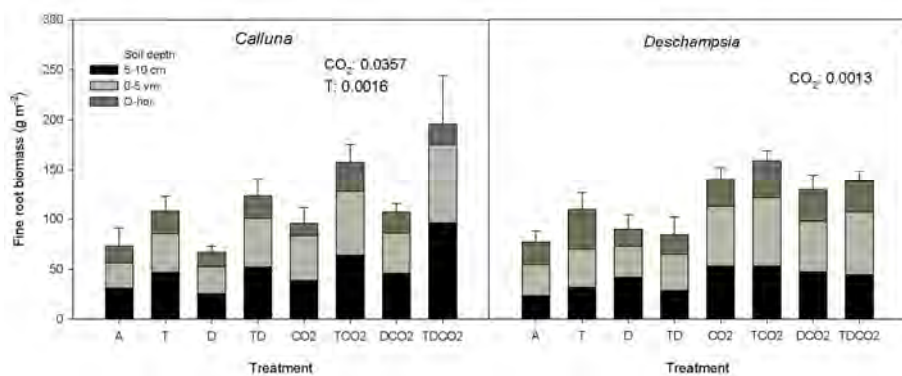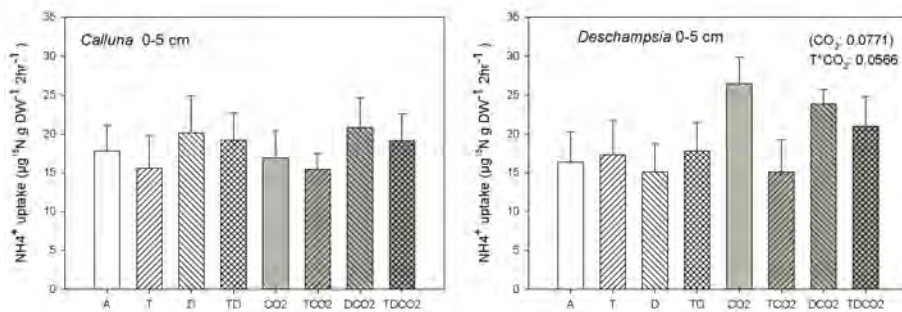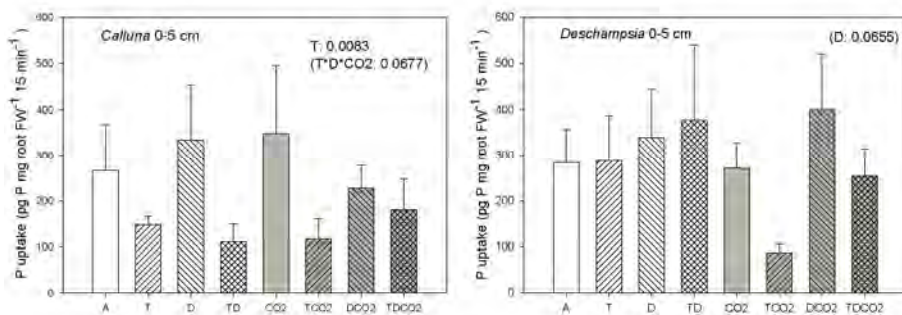
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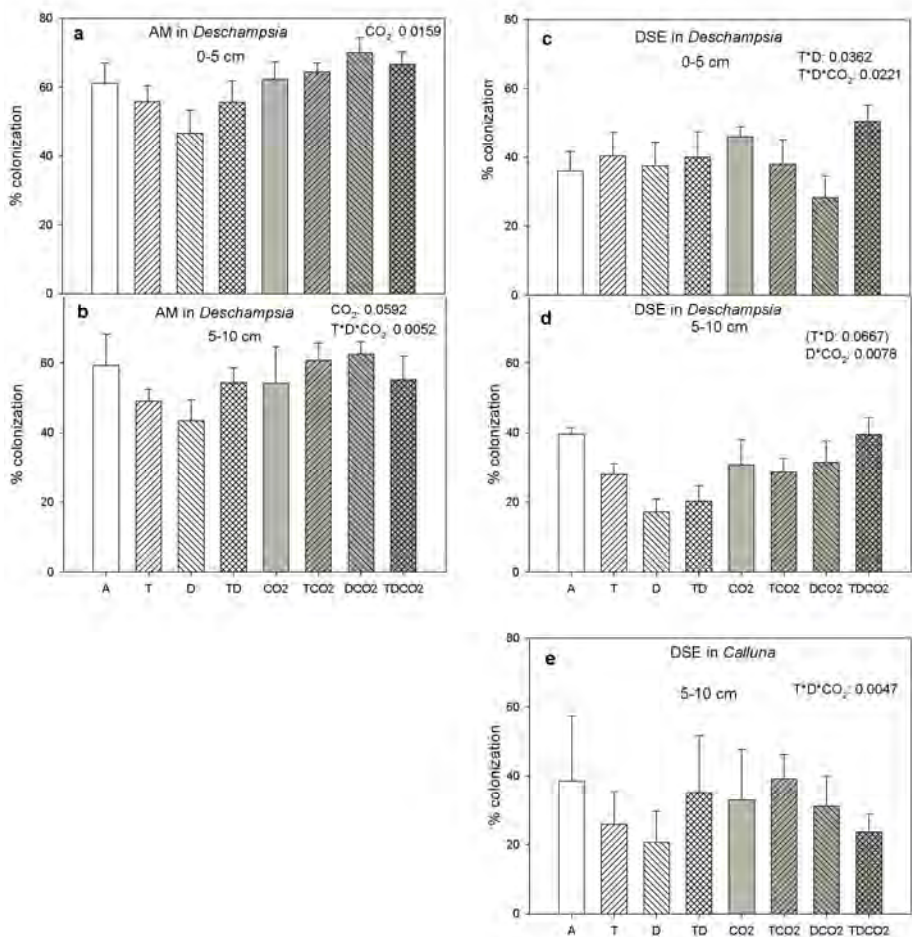**Fig.3**.

Fig. 4.

Fig. 5.

Tabel 1. *Fine root C:N ratio, root N concentration (mg g^{-1}), and plant N pool (mg m^{-2}) for Calluna vulgaris (C) and Deschampsia flexuosa (D). All values are mean±1SE. P values of treatment effects are shown in the last column, and arrows indicate direction of response.*

Para-meter	Species depth cm		A	T	D	TD	CO2	CO2	DCO2	TDCO2	P value
C:N ratio	C	0-5	40.1±2.6	45.9±2.8	43.8±2.3	39.6±3.0	46.6±3.6	48.2±3.1	41.3±2.2	42.3±4.1	↓D: 0.0335
	C	5-10	52.3±3.7	51.4±2.7	48.3±2.6	47.3±3.5	54.2±4.4	56.6±3.4	48.9±2.4	48.8±4.6	↓D: †
	D	0-5	39.7±2.4	45.8±2.6	39.7±2.5	46.7±3.9	48.7±2.6	47.9±1.4	44.9±2.0	45.37±2.1	↑ CO$_2$: 0.049
	D	5-10	50.1±4.2	56.23±5.1	48.2±3.6	47.6±3.2	62.8±4.2	52.2±1.4	54.8±3.6	56.0±4.5	↑ CO$_2$:†
N mg g^{-1}	C	0-5	11.7±0.83	10.1±0.46	10.7±0.5	11.8±0.96	10.0±0.71	9.6±0.63	10.9±0.57	11.3±1.04	↑ D: 0.0458
	C	5-10	8.8±0.79	8.8±0.41	9.5±0.51	9.7±0.71	8.6±0.59	8.0±0.52	9.1±0.53	9.4±0.85	↑ D: †
	D	0-5	11.4±0.62	10.0±0.60	11.5±0.73	10.2±1.16	9.3±0.47	9.4±0.27	10.0±0.45	10.0±0.49	↓ CO$_2$: 0.0386
	D	5-10	9.3±0.65	8.2±0.72	9.6±0.80	9.6±0.85	7.2±0.43	8.5±0.22	8.3±0.53	8.1±0.64	↓CO$_2$:†
N pool mg m^{-2}	C	0-5	279±47	396±54	286±45	536±55	428±38	595±74	437±50	818±171	↑ CO$_2$: 0.0035 ↑ T: 0.0048 ↑ D: 0.0504
	C	5-10	250±59	399±66	245±34	478±86	313±58	507±71	422±69	842±161	↑ CO$_2$: 0.0037 ↑ T: 0.0003 ↑ D: †
	D	0-5	354±64	383±54	362±94	335±81	558±47	642±74	507±21	620±47	↑ CO$_2$<0.0001
	D	5-10	211±31	249±34	381±67	257±50	380±44	450±64	385±39	360±44	↑ CO$_2$: 0.0022 ↑D×CO$_2$: 0.0480

Tabel 2. *Fine root P concentration (mg g^{-1}) and plant P pool (mg m^{-2}) for Calluna vulgaris (C) and Deschampsia flexuosa (D). All values are mean±1SE. P values of treatment effects are shown in the last column, and arrows indicate direction of response of main factors. The treatments are: A (ambient), CO2 (elevated CO$_2$), D (drought) and T (elevated temperature) and all treatment combinations.*

Para-meter	Species depth cm		A	T	D	TD	CO2	CO2	DCO2	TDCO2	P value
P mg g^{-1}	C	0-5	1.34±0.04	1.23±0.06	0.97±0.24	1.50±0.05	1.21±0.11	1.20±0.07	1.39±0.10	1.55±0.11	↑ D: † T×D×CO2:†
	C	5-10	1.15±0.07	1.23±0.10	1.26±0.10	1.42±0.06	1.15±0.12	1.20±0.10	1.24±0.10	1.25±0.09	
	D	0-5	1.23± .11	1.27±0.09	1.12±0.06	1.21±0.14	1.25±0.08	1.12±0.06	1.24±0.09	1.18±0.05	
	D	5-10	1.03±0.02	1.08±0.04	1.1±0.08	1.08±0.11	1.11±0.08	1.08±0.10	1.10±0.06	1.08±0.12	
P pool mg m^{-2}	C	0-5	42±8	47±6	38±7	72±11	51±4	75±10	56±6	102±25	↑ T: 0.0134 ↑CO2: 0.0392
	C	5-10	43±16	62±11	30±5	73±15	45±11	80±17	62±11	114±22	↑ T: 0.0006 ↑CO2: 0.0250
	D	0-5	44±8	51±8	45±15	60±15	74±4	76±8	63±5	73±6	↑ T: 0.0002 ↑CO2: 0.0363 T×CO2: 0.0019
	D	5-10	19±5	38±8	51±11	43±16	58±6	59±12	54±8	48±7	↑CO2: 0.0257 D×CO2: †

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