

# Nitrogen fertilisation reduces sink strength of poplar ectomycorrhizae during recovery after drought more than phosphorus fertilisation

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## Abstract

**Background and aims** Drought reduces the carbon (C) flux from leaves (source) to mycorrhizal roots (sink); however, during recovery from drought, C flux exceeds the levels observed in irrigated controls. This process could be source- or sink-controlled. We studied this source–sink relationship in an agronomically used poplar clone grown at different levels of nitrogen (N) and phosphorus (P) fertilisation as used in silvoarable agroforestry systems.

**Methods** We conducted a fully factorial pot experiment combining four fertiliser and two drought regimes. Gas exchange and chlorophyll and flavonol indices were regularly monitored. One week after rewatering, we

performed  $^{13}\text{C}$  pulse labelling. At harvest, enzyme activities of ectomycorrhizal root tips were determined. **Results** After one week of recovery, we observed an excess in C allocation to ectomycorrhizae (ECM) in non-N-fertilised treatments. However, net photosynthesis only recovered to the level of continuously irrigated controls. Drought increased chitinase, cellulase, phosphatase and peptidase activities, but the latter only in N-fertilised treatments.

**Conclusions** We add evidence that the allocation of recently assimilated C is most likely sink-controlled. Less C allocation to recovering ECM supplied with fertiliser may be either due to better nutritional status and hence higher stress tolerance, or due to partitioning between above and below-ground sinks.

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## Introduction

Future climate models predict warmer and drier summers in the temperate regions and more variability in precipitation (Field 2012). The distribution of assimilated  $\text{CO}_2$  in plants is a source–sink relationship, where photosynthesis in the chloroplasts is the source, building up a pool of non-structural carbohydrates (NSC, mostly sugars and starch), which are used in sinks such as growth (building up structural carbohydrates), defence and the maintenance of primary and secondary metabolism (Hartmann and Trumbore 2016). During water

shortage, this C flux from source to sinks can be diminished, whereas during recovery from drought—when there is ample water again—the flux can exceed the rates under optimal water supply (Hagedorn et al. 2016). Several studies have addressed the issue of whether this flux is under source or sink control. As drought reduces primary productivity, it was formerly assumed that C balance under drought is controlled by restricted source activity rather than by changes in sink strength (Guillemot et al. 2015; Sala et al. 2012). This was inferred by the fact that stomata close under water shortage and thereby reduce gas exchange in leaves (Brodrribb and McAdam 2011; McDowell et al. 2008). However, owing to loss of turgor during dry periods, meristematic activity (i.e. growth, a C sink) is reduced earlier than CO<sub>2</sub> uptake, leading to an accumulation of NSC in early phases of drought (Muller et al. 2011). Additionally, direct environmental sink control has been proposed (Hagedorn et al. 2016; Körner 2003), although no clear evidence of this has yet been found, probably because of other processes that could be limiting under drought, such as nutrient availability, temperature or cell turgor (Körner 2015).

Even less understood are the mechanisms regulating C fluxes during recovery after drought. Plant C dynamics are often inferred by measuring NSC pools of the plant. Thereby it is assumed that fluctuations in these pools are only due to their utilisation. However, storage of C in plants (NSC pools) may be adaptive and respond to environmental stimuli by basically shifting C from one pool to another (Hartmann and Trumbore 2016). By applying <sup>13</sup>C<sub>2</sub>O pulse labelling to trace recently assimilated CO<sub>2</sub>, Hagedorn et al. (2016) showed that beech trees direct recent assimilates preferentially to ectomycorrhizae (ECM) after release from drought. Further, this study observed faster recovery of soil respiration than of photosynthesis and concluded that metabolic activity in the rhizosphere is not source-driven. Moreover, C fluxes to roots and ectomycorrhizal fungi after drought not only recovered, but were actually higher than before the drought and thus compensated for previous effects of stress, leading to the hypothesis that the reactivation of root metabolism is important for ecosystem resilience to drought.

Mycorrhizae are the organs by which most plants including trees acquire nutrients and water. Poplars are among the most commonly planted trees in Central European agroforestry systems (Reisner et al. 2007). They can form both ectomycorrhizal and arbuscular

mycorrhizal associations. While arbuscular mycorrhizae (AM) are very common (80% of all plants), ectomycorrhizal symbiosis dominates in forests of the northern hemisphere (Finlay 2008; Smith and Read 2010). Temperate and boreal forest soils contain most nutrients (especially nitrogen and phosphorus) in organically bound form and trees, therefore, depend on nutrient mobilisation by associated or free-living soil microorganisms (LeBauer and Treseder 2008; Maracchi et al. 2005; Smith and Read 2010). This mobilisation is accomplished predominantly by fungal enzymes (Kjøller and Struwe 2002). For the nutrition of forest trees, the enzyme activities of their ECM play a major role (Perez-Moreno and Read 2000; Tibbett and Sanders 2002).

In the present study, we investigated the effect of drought and different levels of nitrogen (N) and phosphorus (P) on the distribution of recently assimilated carbohydrates to ECM of the poplar clone ‘Rochester’ (*Populus maximowiczii* × *P. nigra* var. *plantierensis*), which is widely used in short-rotation coppice agroforestry. We used stable isotope labelling to test whether C fluxes during recovery after drought are sink-controlled as in beech (Hagedorn et al. 2016) and if this source–sink relationship is modulated by agriculturally relevant levels of nutrients. We tested the following alternative hypotheses about the source–sink control: (I) If source activity controls the C distribution after drought, an initial excess of photosynthesis is expected, followed later by a C signal in ECM, and (II) if sink activity controls C distribution after drought, an initial C signal in ECM is expected, followed later by a peak of photosynthetic activity. We suggest that C allocation to recovering ECM will be lower in fertilised treatments, as a better nutritional state of the plant may mitigate drought stress. Low N fertilisation has been shown to increase water use efficiency (WUE) of woody plants under drought, thus compensating for drought stress (Wu et al. 2008). By contrast, fertilisation with P has been found to increase drought tolerance mainly of non-woody AM plants (Nelsen and Safir 1982). We further expect enzyme activities of ECM in recovering plants to be generally higher due to the increased nutrient demand needed for regrowth of tissues lost during drought and for rebalancing nutrient stoichiometry (Gessler et al. 2016). Moreover, mineral fertilisation may decrease the enzymatic activity of ECM as there is no need to break down organic compounds in order to take up nutrients. To address these issues, we conducted an experiment to test the effects of N and P fertilisation

on ectomycorrhizal enzyme activities and C source–sink processes of poplar species during recovery from drought via the tracing of labelled photosynthates.

## Material and methods

### Plant material and growth conditions

We used clonal plants because approximately 50% of the variation in plant traits occurs at the level of individuals (Garnier et al. 2001; Jung et al. 2010). We obtained cuttings of the intersectional poplar variety ‘Rochester’ (*P. maximowiczii* × *P. nigra* var. *plantierensis*, institute no. 138/49, registration no. 091960 01001 4), from the Bavarian Office for Forestal Seed and Plant Breeding (ASP) in Teisendorf, Germany. Before planting, the cuttings were first washed in distilled water, dosed with a few drops of the wetting agents Tween 20 and Tween 80. Subsequently, they were surface-sterilised in 30% (v/v) H<sub>2</sub>O<sub>2</sub> for 5 min and finally rinsed with freshly distilled water. Cuttings were grown in 2.5-L pots (11 × 11 × 21 cm) that had been sterilised with ethanol (80%) and contained a sterile mixture of 1/3 farmland soil and 2/3 vermiculite (Table 1). We chose vermiculite to optimise the growth conditions in the clayey soil and because the ability of vermiculite to sequester phosphorus is rather low (Brix et al. 2001; Hayes et al. 2000; Ramulu et al. 1967). This soil mixture was autoclaved in batches of 15 kg at 134 °C for 120 min to standardise future fungal and microbial growth. The soil had been taken close to an agroforestry plot (silvoarable short-rotation coppice alley cropping) at the research farm Scheyern (Germany, 48°29′46.5″N 11°26′52.3″E) and was characterised as stagnic Cambisol (WRB). The two upper horizons were collected down to ca. 30 cm (Table 2). The surface of the soil mixture was covered with 2 cm of fire-dried and autoclaved quartz sand and a dense polypropylene fabric of 1–2 mm thickness (pond fleece), leaving free only the top bud of the cuttings.

**Table 1** Concentrations [mg kg<sup>-1</sup> dry matter] of plant available nutrients and total N in fresh soil and the soil mixture used for the experiment

Material	Phosphorus	Ammonium	Nitrate	Nitrite	Total N
fresh soil	8.91	0.27	14.95	0.00	11.27
autoclaved soil	9.34	5.43	14.20	0.00	26.58
autoclaved soil mixture	10.09	14.00	13.21	0.00	34.79

Pots were transferred to a greenhouse cabin with a 16 h/25 °C day and 8 h/17 °C night regime at 60% relative humidity. Additional illumination (Philips Son-T Agro 400, ca. 373 μmol m<sup>-2</sup> s<sup>-1</sup> PAR at canopy level) was switched on when the light outside was below 15 klux. Plants were watered automatically with deionised (conductivity ≈ 10 μS) water near the maximum holding capacity of the substrate. We defined the maximum water holding capacity as reached when the supplied water began to leak from the bottom of the pots. All leaked water was poured back into the pots to prevent leakage of nutrients. After 11 weeks, established plants were transferred to round 6-L pots (diameter: 24 cm, height: 21.6 cm) containing the same soil mixture with fresh rhizosphere soil (ca. 100 g) added as soil microbial/fungal inoculum. Rhizosphere soil was collected from the top 10 cm at 10 spots at least 9 m distant from each other at a poplar agroforestry plot next to the field the substrate soil had been taken from. Soil crumbs adhering to roots with not more than 1 cm in diameter were shaken off and carefully mixed but not sieved to minimise further disturbance particularly of fungal hyphae. The inoculum was stored overnight at 4 °C and crumbled manually into the planting hole.

As the cuttings sprouted unequally, plants were cut back three weeks after repotting to leave only three buds. The first one that sprouted formed the new shoot. To prevent infestation by thrips during regrowth, the predator mite *Amblyseius cucumeris* was applied. During the experiment, the position of the pots was regularly randomised.

### Treatments

Six weeks after the shoots had been cut back, the plants were subjected in a factorial experiment to the eight combinations of two watering treatments (drought and irrigation) and four fertilisation treatments: no fertilisation (0), addition of 700 mg of phosphorus (K<sub>2</sub>HPO<sub>4</sub>; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) (+P), addition of 290 mg of nitrogen (KNO<sub>3</sub>

**Table 2** Grain-size distribution within the 2 different soil horizons used for the experiment

Horizon	Depth [cm]	Sand [%]	Silt [%]	Clay [%]	Bulk density [ $\text{g cm}^{-3}$ ]
Ap	0–28	18.7	54.6	26.7	1.30
BvSw	28–74	22.6	53.2	24.2	1.68

Ap: ploughed A horizon, BvSw: slack water funnelling brown earth

with 20%  $^{15}\text{N}$ ; Cambridge Isotope Laboratories Inc., Andover, MA, USA) (+N), and addition of both nutrients (+N + P). The amounts of nutrients added were based on the Bavarian guidelines for farmland fertilisation (Wendland et al. 2014). Each pot received 500 mL of a fertiliser solution, except the non-fertilised treatments, which received 500 mL of deionised water.

Directly after fertilisation, half of the pots were subjected to gradual drought by reducing irrigation each week by 25% of the control amount, until the final level of 25% of control irrigation had been reached after three weeks. The volumetric water content in the drought pots was monitored every 20 min with CMP-2 probes connected to a DL-200 data logger (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany). Additional PF-probes (ecoTech Umwelt-Meßsysteme GmbH, Bonn, Germany) were installed in two drought-treated pots to get an approximation of the soil water potential. The control pots were checked manually with a UMP-1 BT probe (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany). After three weeks, most of the drought-treated pots had a volumetric water content of less than 10% (equal to a pF value of approximately  $4 = -10^4$  hPa soil water potential), which was maintained for 24 days. The irrigated pots had a mean volumetric water content of  $23 \pm 1.5\%$  (Fig. 1).

To initiate the recovery phase, the plants from the drought treatments received the same amount of irrigation as control plants. Rewatering took place one week before labelling with  $^{13}\text{CO}_2$  on three successive days, 40, 41 and 42 days after beginning of the drought, one for each labelling batch (14–15 pots). At harvest, one day after labelling, there were six plants left for each treatment combination, except for the treatment combinations +N + P + irrigation and +N + P + drought, for which there remained four replicates.

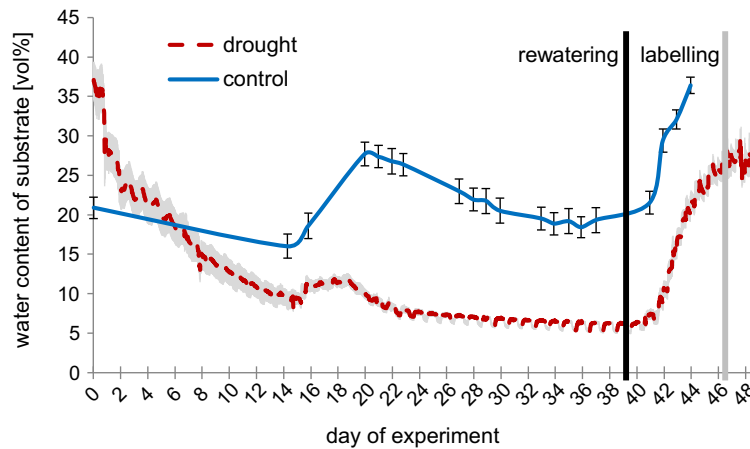
### Labelling

Before labelling, the pots were enclosed in autoclaving bags, tightly sealing the opening around the stem base to

minimise the dilution of  $^{13}\text{CO}_2$  by soil respiration. Inside a separate greenhouse chamber (same conditions as in the growth chamber, except air temperature, which was lowered to control warming inside the tent), an airtight transparent plastic tent (ethylene-tetrafluorethylene) with a volume of approximately  $7.5 \text{ m}^3$  was erected over the pots. Before labelling, the  $\text{CO}_2$  concentration in the tent was decreased to approximately 325 ppm by pumping the air in the tent through soda lime. Labelling started at 09:00 h with 2.6%  $\text{CO}_2$  (99 atom%  $^{13}\text{C}$ ) in  $\text{N}_2$  (Westfalen AG, Münster, Germany) supplied at a flow rate of  $14 \text{ L min}^{-1}$  for 10 min,  $0.7 \text{ L min}^{-1}$  for 50 min and  $0.8 \text{ L min}^{-1}$  for 60 min. After closing the gas valve, plants were allowed to assimilate  $^{13}\text{CO}_2$  for a further 30 min. The use of fans ensured good mixing of the air inside the tent. During the labelling period, air temperature and humidity were measured and the  $^{12/13}\text{CO}_2$  concentration inside the tent was monitored and its  $^{13}\text{C}$  signature was measured with a GC/IRMS-system (Delta Plus; Thermo Fisher, Dreieich, Germany). The mean air temperature was  $20.9 \text{ }^\circ\text{C}$  and the mean relative humidity was 76.3%. During the first 20 min of labelling, the  $\text{CO}_2$  concentration inside the tent rose to  $957 \pm 52$  ppm and the  $^{13}\text{C}$  signature to  $68.2 \pm 1$  atom%  $^{13}\text{C}$ , then increased slowly to  $1016 \pm 64$  ppm and  $76.3 \pm 0.3$  atom%  $^{13}\text{C}$  during the following 110 min and thereafter decreased to  $951 \pm 57$  ppm and  $76.5 \pm 0.4$  atom%  $^{13}\text{C}$  during the last 30 min without further  $\text{CO}_2$  supply.

### Leaf gas exchange measurements

Leaf gas exchange was measured weekly on two consecutive days in the greenhouse using a portable gas exchange measuring system equipped with an  $8 \text{ cm}^2$  clamp-on cuvette (GFS-3000; H. Waltz GmbH, Effeltrich, Germany). Steady-state measurements were performed each week on leaf #9 (the ninth fully developed leaf counted from the apex; six plants per treatment and four plants in the +N + P treatment), mimicking in the cuvette the growing conditions during daytime [ $400 \text{ ppm CO}_2$ ,  $25 \text{ }^\circ\text{C}$  and  $300 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ ]



**Fig. 1** Water content of drought and control pots during the course of the experiment starting on October 8th, 2014. Water content in drought pots (dashed line) was recorded every 20 min using sensors ( $n = 20$ ) connected to a data logger; control pots

(solid line) have been measured manually. Owing to plant growth, irrigation had to be adjusted on day 15 (October 23rd). Error bars indicate  $\pm 1$  SE, the vertical lines indicate the start of rewatering at day 40 (black), and the beginning of  $^{13}\text{C}$  labelling at day 47 (grey)

photosynthetic photon flux density (PPFD)]. The variables used here are net assimilation rates ( $A_n$ ) and instantaneous water use efficiency [WUE, ratio of net photosynthetic  $\text{CO}_2$  uptake to loss of water by transpiration (Pou et al. 2008)].

#### Optical non-destructive assessment of leaf chlorophyll, flavonol and nitrogen status

Indices for chlorophyll content (CHL), epidermal flavonols (FLAV) and the nitrogen balance index (NBI), namely, the CHL/FLAV ratio as an indicator of leaf nitrogen content (Cartelat et al. 2005; Cerovic et al. 2012), were monitored in leaves by non-destructive optical measurements with a leaf-clip sensor, Dualex Scientific + <sup>TM</sup> (ForceA, Orsay Cedex, France). The indices were measured on leaf #9 (or comparable  $\pm 1$ ) once a week. During the first 4 days of recovery from drought, measurements were performed daily. Three measurements were taken on each side (adaxial and abaxial) of the lamina, avoiding veins. The CHL index per leaf was calculated as the mean value of all measurements. As it was shown that the NBI calculated using the sum of FLAV was better correlated to total N in grape leaves than when using only one side (Cerovic et al. 2012), the mean FLAV index per leaf was calculated from the sum of FLAV on the adaxial and abaxial sides of each measuring point. The NBI per measuring point was only calculated when the FLAV index could be measured on both sides per measuring point.

#### Nutrient analysis of soil mixture

Plants were harvested 24 h after labelling. Three replicate soil mixture samples per pot were taken for the determination of nitrogen and phosphorus content. To determine plant-available nitrogen ( $N_{\text{min}}$ ), 5 g of fresh soil mixture was agitated in an overhead shaker with 20 mL of 0.01 M  $\text{CaCl}_2$  solution in glass vials for 45 min and filtered through a pleated filter (Whatman 595  $\frac{1}{2}$ ). Extracts were analysed wet chemically in an automatic analyser with continuous flow air segmented injection (SA20/40, Type 5100; Skalar Analytical, Breda, The Netherlands). Ammonium forms a complex with salicylate, which can be measured at 660 nm, while nitrate is reduced to nitrite in a cadmium column to a diazo complex and measured at 540 nm. Nitrite was measured directly at 540 nm (VDLUFA 1991).

To determine plant-available phosphorus ( $P_{\text{CAL}}$ ), 2.5 g of fresh soil mixture was placed on an overhead shaker with 50 mL of CAL solution (calcium-acetate-lactate) in Falcon tubes for 90 min, filtered and analysed photometrically after colour reaction with molybdenum (VDLUFA 1991). The determination of plant-available P using the CAL method was previously shown to yield results that are more predictive of plant yield than the  $P_i$ , Colwell, Bray 1 and Truog tests (Kumar et al. 1994).

#### Mycorrhiza sampling

Plant roots were washed carefully and samples of fine roots were taken randomly from the root ball.



As not the entire root system was analysed, only relative values were used for statistical analyses and descriptions. Staining of fine roots with ink (Sheaffer black; Sheaffer Pen Corporation, Shelton, CT, USA) and vinegar (Vierheilig et al. 1998) revealed no AM colonisation. Ectomycorrhizal root tips (ECM tips) were separated into morphotypes (Agerer 1991) under a dissecting microscope. Vital ECM tips were collected into tin capsules on dry ice for later stable isotope analyses and on wet filter paper for exoenzyme activity measurement. Additional ECM tips were used for morphotype identification based on their DNA sequence (see method S1). ECM with a crumpled surface, desiccated appearance and no white vascular tissue inside were defined as non-vital. Root tips without a clearly visible hyphal mantle were considered as non-mycorrhizal (no functional ECM) and not sampled. Twenty-one vital ECM tips were selected from each sample for enzyme activity measurements. The number of ECM tips of each morphotype was chosen according to the relative abundance of the respective morphotype in the sample, but was not lower than three. Based on these 21 ECM tips, the weighted mean was calculated and considered as representing the exoenzymatic potential of the ectomycorrhizal community in the sample. Further ECM tips were placed in tin capsules dried at 70 °C until weight constancy and analysed in an Isotope Ratio Mass Spectrometer (IRMS, type delta V Advantage), coupled with an Element Analyser (Euro EA).

Enzymatic activities were measured in microplates (Pritsch et al. 2011). Seven substrates bound to 4-methylumbelliferone (MU) or aminomethylcoumarin (AMC) and ABTS [diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)] were used: l-leucine 7-AMC (Leu-AMC) for the detection of leucine aminopeptidase (EC 3.4.11.1), 4-MU  $\beta$ -D-xylopyranoside (MU-X) for xylosidase (EC 3.2.1.37), 4-MU- $\beta$ -D-glucuronide hydrate (MU-GU) for glucuronidase (EC 3.2.1.31), 4-MU  $\beta$ -D-cellobioside (MU-C) for cellobiohydrolase (EC 3.2.1.91), 4-MU N-acetyl- $\beta$ -glucosaminide (MU-NAG) for chitinase (EC 3.2.1.14), 4-MU  $\beta$ -D-glucopyranoside (MU-G) for  $\beta$ -glucosidase (EC 3.2.1.3), 4-MU phosphate-free acid (MU-P) for phosphatase (EC 3.1.3.2) and ABTS for laccase (EC 1.10.3.2) activities. In brief, individual ectomycorrhizal root tips were placed in wells of 96-well filterplates

(AcroPrep™ Advance 96 Filter Plate, 30–40  $\mu$ m PP/PE, 350- $\mu$ L wells, NTRL; Pall Corporation, Ann Arbor, MI, USA) and incubated with the respective substrate. The solution with the incubated substrate was transferred to a black 96-well plate (FluoroNunc™ F96 MicroWell™ Plate) by applying a vacuum under the filter plate with a vacuum manifold (Pall Corporation, Ann Arbor, MI, USA). From fluorescence and absorbance measurements, the quantity of substrate turned over was calculated and expressed as  $\text{pmol mm}^{-2} \text{min}^{-1}$ .

### Leaf sampling

At harvest, leaves (including the petiole) were separated from the shoot and dried in paper bags at 70 °C until weight constancy. After drying, all leaves per plant were pre-ground with a rotor mill (Fritsch Rotor Speed Mill Pulverisette 14; Fritsch GmbH, Idar-Oberstein, Germany) equipped with a 0.5-mm sieve (Fritsch GmbH, Idar-Oberstein, Germany) at 8000 rpm and finally homogenised with the same mill equipped with a 0.08-mm sieve (Fritsch GmbH, Idar-Oberstein, Germany) at 10,000 rpm. From each sample, three technical replicates of ca. 1.5 mg were analysed for  $^{13}\text{C}$  and  $^{15}\text{N}$ , total N and total C content in an Isotope Ratio Mass Spectrometer (IRMS, type delta V Advantage; Thermo Fisher, Dreieich, Germany), coupled with an Element Analyser (Euro EA; Eurovector, Milan, Italy). Leaf P content was analysed via inductively coupled plasma atomic emission spectrometry (ICP-AES, Cirovision; Spectro, Kleve, Germany) after digestion of homogenised leaf material with nitric acid.

### Stable isotope analysis

$^{15}\text{N}$  and  $^{13}\text{C}$  abundances were determined with an IRMS (Delta V Advantage) coupled with an Elemental Analyzer (Euro EA). A laboratory standard (acetanilide) was used at regular intervals of every measurement sequence. It was also used at different weights to determine the isotope linearity of the system. The laboratory standard itself is calibrated against suitable international isotope standards (IAEA; Vienna) for  $^{15}\text{N}$  and  $^{13}\text{C}$ . Several international isotope standards were also part of every sequence. For  $^{13}\text{C}$ , these were IAEA 309 A and IAEA 309 B. For  $^{15}\text{N}$ , these were IAEA N2, USGS 41, USGS 26 and USGS 32. For samples with

high  $^{15}\text{N}$  abundances, laboratory standards between 6 and 15 atom% were used. These standards were formerly calibrated with standards from Fischer Analysen Instrumente (Leipzig, Germany).

### Statistical analysis

Values in this article are given as mean  $\pm$  standard error (SE) if not indicated differently. All statistical analyses were conducted using R (R Core Team 2016). The effects of the treatments on measures of growth and stable isotope abundance in ECM and leaves of the plants were analysed using three-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) test. In the analysis of the influence of the treatments on  $^{13}\text{C}$  abundance in ECM, leaf area was used as a covariate to take into account differences in photosynthetic capacity between plants. To test the influence of irrigation on the distinct fertiliser combinations, one-way ANOVA was used on the respective subsets of data. Enzymatic activity patterns were analysed with ANOVA. To ensure normally distributed residuals and homogeneity of variance, the dependent variables were log- or square-root-transformed, if necessary. Data from optical non-destructive assessment of leaf chlorophyll, flavonol and nitrogen status as well as gas exchange measurements and soil moisture probes data were analysed using mixed effect models. The R package nlme (Pinheiro et al. 2014) was used and individual plants were considered as a random factor, namely, we used random intercepts for plants. Random slopes per day were not used because this did not result in a significant improvement of the model. Data were transformed to meet model assumptions, if necessary.

## Results

At harvest (49 days after fertilisation and onset of drought), the N content in leaves was dependent on N fertilisation and the irrigation regime. In continuously irrigated plants, leaf N content was rather low (+N:  $0.65 \pm 0.01\%$ , -N:  $0.57 \pm 0.01\%$ ). In leaves of recovering plants, N content was slightly higher in non-N-fertilised treatments ( $0.82 \pm 0.08\%$ ), but much higher in N-fertilised treatments ( $1.33 \pm 0.05\%$ ; treatment  $\times$  N:  $p < 0.001$ ). More  $^{15}\text{N}$  from the fertiliser was found in leaves of non-P-fertilised plants ( $10.38 \pm 0.34$  atom%) than in P-fertilised plants ( $9.54 \pm 0.42$  atom%;

$p = 0.140$ ). Leaf P content of P-fertilised plants ( $4.03 \text{ mg g}^{-1}$ ) was 2.2 times higher than in non-P-fertilised plants ( $1.84 \text{ mg g}^{-1}$ ;  $p < 0.001$ ). This resulted in low N:P ratios when only P was fertilised (below 1.7) and high N:P ratios when only N was fertilised (over 5.26). Leaves of drought treated plants generally had higher N:P ratios than leaves of continuously irrigated plants (Table 3).

Following nitrogen balance index (NBI) of leaf #9 over time revealed that fertilised N was taken up quickly at the beginning of the experiment in continuously irrigated plants, whereas uptake was retarded in drought treatments. Fertilisation with N increased NBI during two weeks after N application to values 1.6–2 times higher than in non-N-fertilised plants (Fig. 2). While NBI of leaf #9 in irrigated plants declined within the following three weeks to values in the range of plants without N application, NBI and hence N nutrition in drought-treated plants remained significantly higher until the end of the experiment (significant irrigation  $\times$  N fertilisation interaction during severe drought and during recovery:  $p < 0.01$ ; Table 4).

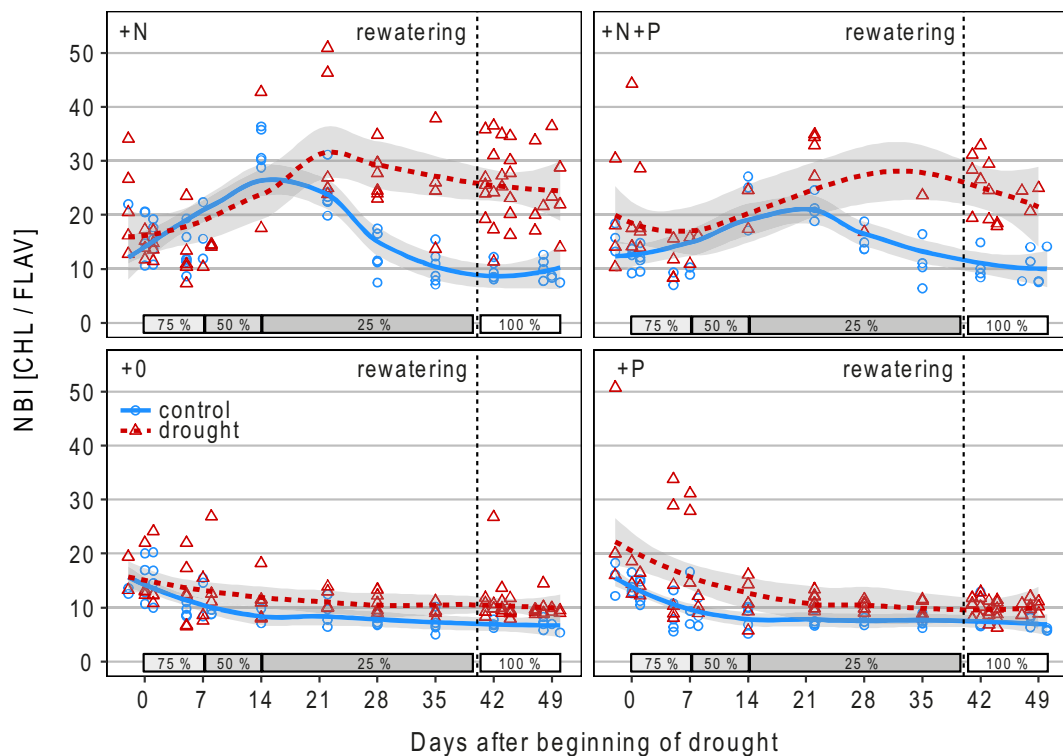
The low N content in leaves at harvest and the fast decrease of NBI in irrigated plants two weeks after fertilisation reflected the rapid depletion of N from the soil mixture. Respectively, higher N contents and NBI in drought treated recovering plants showed impeded N-uptake during drought. At harvest, the supplied nitrogen was depleted from  $18.8 \pm 3.8$  and  $80.7 \pm 3.8 \text{ mg/kg}$  dry soil mixture in non-N- and N-fertilised treatments, respectively, to  $1.6 \pm 0.2 \text{ mg/kg}$  at harvest in all treatments. Thus, 4.6 times more N was taken up in N-fertilised treatments. Phosphorus content in the substrate at the beginning of the experiment was  $53.1 \pm 1.2 \text{ mg/kg}$  in non-P- and  $156.8 \pm 1.2 \text{ mg/kg}$  in P-fertilised treatments. Thus, 3.4 times more P was taken up in P-fertilised treatments (Table 5). The soil mixture in the pots was entirely penetrated by roots.

Numerous significant irrigation  $\times$  N interactions ( $p < 0.001$ , Table 3) showed that the increase in above-ground plant growth and thereby activities of above-ground C sinks due to N fertilisation were much higher in the irrigated treatments. There were also three significant N  $\times$  P interactions ( $p < 0.05$ ): in the absence of N, P addition reduced leaf biomass and increased shoot water content and leaf area, whereas the opposite was observed in the presence of N (Table 3). However, soil moisture, stomatal conductance and WUE were not significantly altered by fertilisation regime, indicating

**Table 3** Mean values ± standard errors for traits of *P. maximowiczii* × nigra clone ‘Rochester’ at harvest, one week after rewatering. The treatments are irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP), and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Below: Analysis of variance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on selected traits of *P. maximowiczii* × nigra clone ‘Rochester’. Significant results ( $p < 0.05$ ) are in bold

Treatment	Leaf N:P ratio		growth increment		Shoot biomass		Leaf Biomass		Shoot height		Leaf area		Shoot water content [%]		Leaf water content [%]		Leaf area		
	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	
C0	2.86	0.09	7.3	0.4	8.2	0.8	12.6	0.8	79.9	3.1	1320	103.4	51.3	2.4	58	0.7	1320	103	
CN	5.26	0.46	9.6	0.2	14.3	0.5	20.5	0.9	112.2	4.5	2063	84.4	57.4	1.7	57.7	0.7	2063	84	
CNP	2.4	0.08	8.7	0.2	14.2	0.8	20.5	0.5	120.8	8.9	2014	69.4	56.8	0.6	58.9	0.5	2014	69	
CP	1.25	0.06	7.5	0.7	6.7	0.5	10.2	0.5	75.8	3.4	1084	57.8	56.4	0.6	60.1	0.7	1083	58	
T0	4.04	0.29	6.6	0.2	5.6	0.4	7.5	0.5	74	3.6	893.8	56.8	57.7	0.6	62.7	0.6	894	57	
TN	5.76	0.18	7.4	0.2	7	0.5	7.2	0.7	87.7	1.8	956.3	62	64.7	1.1	66.1	2	956	62	
TNP	4.11	0.61	7.3	0.2	7	0.4	8	0.1	83	3.5	1057	29.1	64.5	0.6	66.4	0.3	1057	29	
TP	1.68	0.16	6.1	0.3	4.2	0.3	5.4	1.1	68.8	3	666.4	136.5	61.2	1	67.7	3.2	666	137	
Source of variation	df	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Irrigation	1	40.71	<0.001	24.38	<0.001	128.5	<0.001	267.8	<0.001	34.61	<0.001	133.9	<0.001	46.89	<0.001	11.43	0	24.38	<0.001
N-fert.	1	172.8	<0.001	26.53	<0.001	113.6	<0.001	94.44	<0.001	87.88	<0.001	74.57	<0.001	20.23	<0.001	0.2	0.7	26.53	<0.001
P fert.	1	192	<0.001	1.25	0.27	5.46	0.025	4.14	0.049	1.04	0.315	3.63	0.065	5.57	0.024	0.04	0.8	1.25	0.27
Irrig x N	1	0.26	0.61	1.49	0.23	19.78	<0.001	57.84	<0.001	11.07	0.002	26.03	<0.001	0.95	0.336	0.05	0.8	1.49	0.23
Irrig x P	1	2.28	0.14	0.07	0.797	0.06	0.814	0.3	0.589	1.12	0.298	0.33	0.566	0.13	0.724	0.78	0.4	0.07	0.797
N x P	1	7.77	0.009	0.1	0.76	3.61	0.065	6.08	0.019	1.23	0.275	4.34	0.044	6.19	0.018	1.09	0.3	0.1	0.76
Irrig x N x P	1	4.72	0.037	0.19	0.19	0.08	0.785	0.07	0.793	0.76	0.389	0.32	0.577	0.27	0.607	0.25	0.6	0.19	0.19
Residual	36																		





**Fig. 2** NBI (nitrogen balance index: CHL/FLAV) indices in leaf #9 from the apex of *P. maximowiczii* × *nigra* clone ‘Rochester’ during the experiment in irrigated (open circles) and drought-stressed plants (open triangles) separated by fertilisation regime

(+0 = no fertilisation, +N = N fertilisation, +P = P fertilisation, +N + P = N and P fertilisation). Curves show LOESS fits with 95% confidence interval (grey shading) of irrigated (solid line) and drought-stressed (dashed line) plants

that bigger plants did not experience differential drought stress. Growth increment, shoot biomass, leaf biomass, shoot height and leaf area were significantly positively correlated among each other. Water content of leaves and shoots was negatively correlated with the above-mentioned variables and among each other they were significantly positively correlated (Table S2).

Despite effects of N fertilisation on leaf nutrition and plant growth, the fertilisation regime did not significantly affect net assimilation ( $A_n$ ) and WUE. During severe drought with 25% irrigation compared to the control,  $A_n$  and hence source activity (Fig. 3)a was significantly reduced by the irrigation regime ( $p < 0.05$ ), while WUE (Fig. 3)b was significantly increased ( $p < 0.001$ ). During recovery (100% irrigation of the control), the WUE of recovering plants was still significantly higher than that of control plants ( $p < 0.05$ ), but there was no significant difference in  $A_n$  anymore ( $p = 0.350$ ).

One day after labelling  $^{13}\text{C}$  abundance in leaves (C-sources and C-sinks when still growing) was lower than in ECM.  $^{13}\text{C}$  abundance was significantly higher in leaves of plants recovering from drought ( $227.0 \pm 16.8\%$  V-PDB)

than in continuously irrigated plants ( $178.4 \pm 9.5\%$  V-PDB;  $p < 0.05$ ). Overall C content was higher in leaves of recovering plants ( $43.8 \pm 0.2\%$  vs.  $42.8 \pm 0.3\%$ ;  $p < 0.001$ ) and in leaves of plants fertilised with N ( $44.1 \pm 0.2\%$  vs.  $42.6 \pm 0.3\%$ ;  $p < 0.001$ ).

The below-ground sinks (ECM tips) of plants recovering from drought showed higher  $^{13}\text{C}$  abundances (C allocation) than ECM tips from irrigated treatments ( $p < 0.005$ ). This effect was highest in treatments without N fertilisation ( $555.6 \pm 99.0$  vs.  $209.5 \pm 38.0\%$  V-PDB,  $p < 0.005$ ; Fig. 4). ECM tips of recovering plants that were fertilised with nitrogen did not show significant differences in  $^{13}\text{C}$  abundances ( $388.5 \pm 70.5\%$  V-PDB) compared to ECM tips of irrigated plants ( $312.0 \pm 87.6\%$  V-PDB). The non-fertilised treatment showed the greatest difference in  $^{13}\text{C}$  abundance between ECM tips of irrigated and recovering plants. A significant ( $p < 0.05$ ) ordinal irrigation × P fertilisation interaction showed that ECM tips of recovering plants received more C when not fertilised with P (Table 6). There was no significant effect of fertilisation on  $^{13}\text{C}$  abundance among the ECM tips of recovering and control plants.

**Table 4** Effects of irrigation, nitrogen (N) and phosphorus (P) fertilisation on nitrogen balance index (NBI), net photosynthesis ( $A_n$ ) and instantaneous water use efficiency (WUE) in leaf #9 from the apex of *P. maximowiczii* × *nigra* clone ‘Rochester’ during

severe drought (25% irrigation of control plants for 32 days) and during recovery (100% water for 7 days). *p*-values obtained by linear mixed effects models are shown. Significant results ( $p < 0.05$ ) are in bold

Time	Source of variation	NBI	$A_n$	WUE
During severe drought (25% irrigation)	Date	<b>&lt;0.001</b>	<b>0.003</b>	0.247
	Irrigation	<b>&lt;0.001</b>	<b>0.036</b>	<b>&lt;0.001</b>
	N-fertilisation	<b>&lt;0.001</b>	0.35	0.389
	P fertilisation	0.359	0.31	0.066
	Irrig. x N-fert.	<b>0.005</b>	0.388	0.287
	Irrig. x P fert.	0.543	0.971	0.402
	N-fert. x P fert.	0.663	0.415	0.138
	Irrig. x N x P	0.533	0.876	0.708
During recovery (100% irrigation)	Date	0.157	<b>&lt;0.001</b>	<b>0.008</b>
	Irrigation	<b>&lt;0.001</b>	0.35	<b>0.011</b>
	N-fertilisation	<b>0.015</b>	0.697	0.436
	P fertilisation	0.658	0.093	0.868
	Irrig. x N-fert.	<b>&lt;0.003</b>	0.642	0.624
	Irrig. x P fert.	0.583	0.687	0.587
	N-fert. x P fert.	0.924	0.238	0.911
	Irrig. x N x P	0.966	0.673	0.186

Drought reduced the relative number of below-ground sinks. At harvest, double the amount of non-vital ECM tips were found in the drought treatments ( $40.4 \pm 5.5\%$  relative abundance) compared to the irrigated treatments ( $21.1 \pm 3.2\%$  relative abundance;  $p < 0.01$ ). There was no significant difference in the degree of mycorrhization ( $72.6 \pm 2.9\%$  mycorrhizal root tips) or in the abundance of any of the species/morphotypes among the treatments. *Sphaerospora brunnea* was the dominant ectomycorrhizal species ( $40.8 \pm 3.0\%$  overall relative abundance) identified from three out of five morphotypes. The fourth morphotype turned out to represent two macroscopically similar taxa: *Tomentella ellisii* and *Otidea tuomikoskii* ( $1.0 \pm 0.7\%$  overall relative abundance). The fifth morphotype did not yield an evaluable ITS sequence ( $0.2 \pm 0.1\%$  overall relative abundance).

In ECM tips of plants recovering from drought, activities of xylosidase, glucuronidase and cellobiohydrolase did not show significant differences among treatments. Laccase activity was not detectable and hence omitted from further analyses. Yet, activities of chitinase,  $\beta$ -1,4-glucosidase (both degrading cell walls) and phosphatase (P mobilising) were significantly higher in ECM tips of plants recovering from drought ( $83.5 \pm 6.2$ ,  $66.2 \pm 5.9$

and  $31.7 \pm 5.3$  pmol mm<sup>-2</sup> min<sup>-1</sup>, respectively) compared with continuously irrigated treatments ( $63.7 \pm 4.3$ ,  $48.9 \pm 4.0$  and  $20.2 \pm 2.0$  pmol mm<sup>-2</sup> min<sup>-1</sup>, respectively). The effect of drought on the activity of the N mobilising enzyme leucine aminopeptidase was significantly different depending on nitrogen fertilisation. In N-fertilised treatments, ECM tips of plants recovering from drought showed significantly higher activity ( $185.6 \pm 47.5$  pmol mm<sup>-2</sup> min<sup>-1</sup>) than tips from irrigated treatments ( $69.1 \pm 21.7$  pmol mm<sup>-2</sup> min<sup>-1</sup>), while in treatments without N addition, the activity of recovering ECM tips was decreased ( $91.9 \pm 21.1$  pmol mm<sup>-2</sup> min<sup>-1</sup>) compared with that in continuously irrigated treatments ( $146.9 \pm 30.7$  pmol mm<sup>-2</sup> min<sup>-1</sup>; Fig. 5, Table 7). So ectomycorrhizae of recovering plants had a higher potential to release nutrients from soil organic matter. All enzyme activities were positively correlated among each other (Table S3).

## Discussion

Net photosynthesis ( $A_n$ , source activity) was reduced by drought and recovered after rewatering to the level of continuously irrigated plants, but did not go to excess. A

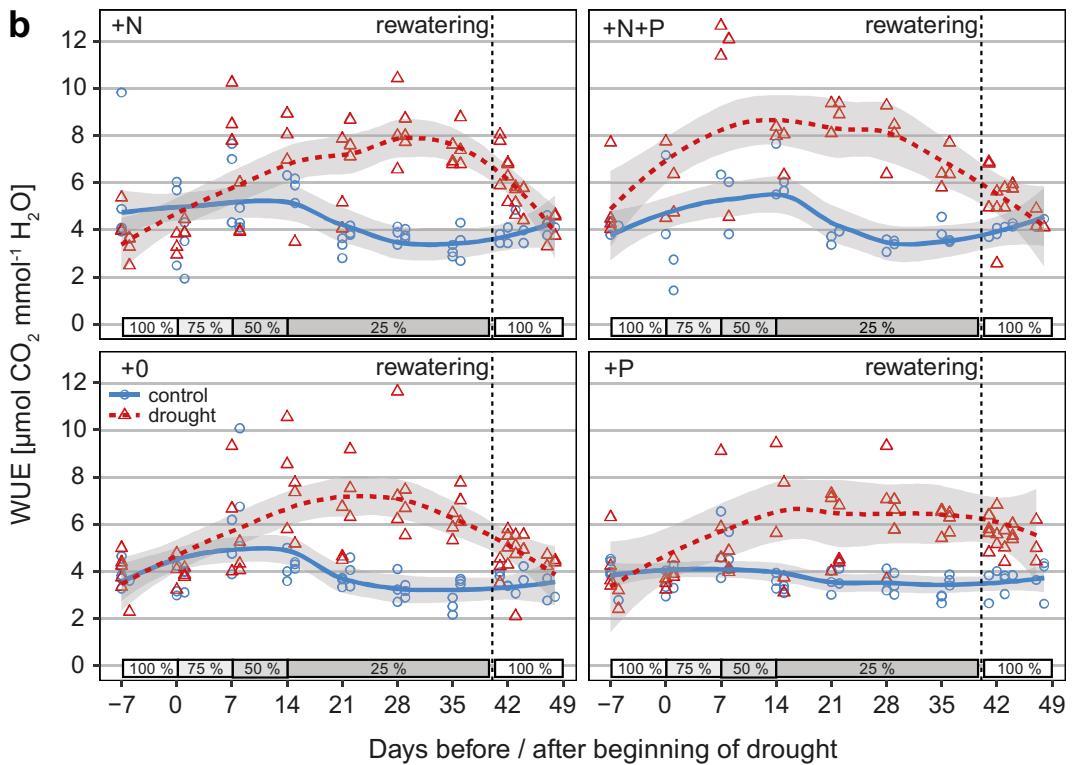
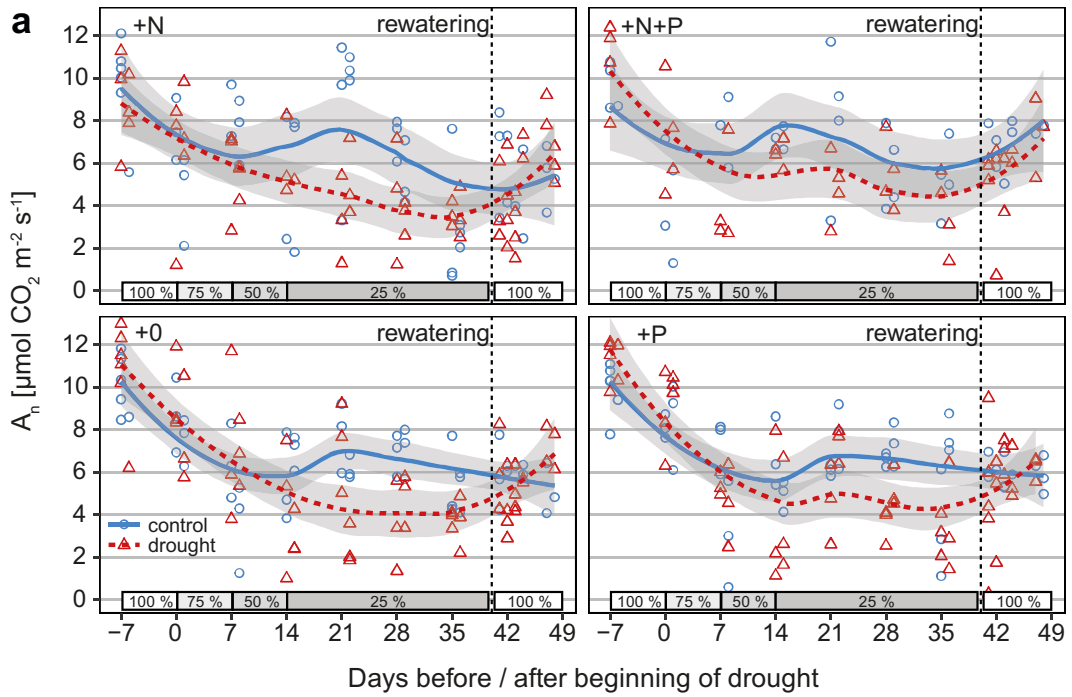
**Table 5** Mean values [mg/kg dry soil mixture]  $\pm$  standard error of N and P contents in the soil mixture before and after fertilisation as well as at harvest. Values for nutrient contents after fertilisation result from the addition of respective nutrient amounts to the values in the soil mixture before fertilisation. The treatments are irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser

(CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Below: Analysis of variance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on N and P contents in the soil mixture at harvest of *P. maximowiczii*  $\times$  nigra clone ‘Rochester’. Significant results ( $p < 0.05$ ) are in bold

Treat-ment	before fertilisation				after fertilisation (calculated)				at harvest			
	N		P		N		P		N		P	
	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se
C0	18.80	3.84	53.10	1.20	18.80	3.84	53.10	1.20	1.01	0.10	12.87	1.19
CN					80.66				2.13	0.84	12.40	1.45
CNP							156.84		1.90	0.55	22.16	2.08
CP					18.80				1.73	0.29	12.16	0.97
T0							53.10		1.79	0.63	15.04	0.75
TN					80.66				1.35	0.17	14.46	0.88
TNP							156.84		1.17	0.10	20.90	2.17
TP					18.80				1.73	0.28	24.88	2.49
Source of variation	df								F	p	F	p
Irrigation	1	initial conditions were equal			calculated values				0.97	0.330	1.90	0.177
N-fert.	1								0.01	0.907	3.18	0.083
P fert.	1								1.62	0.210	49.47	<b>&lt;0.001</b>
Irrig x N	1								0.33	0.571	0.01	0.911
Irrig x P	1								0.14	0.713	1.63	0.210
N x P	1								0.94	0.337	0.15	0.705
Irrig x N x P	1								1.89	0.177	0.19	0.666
Residual	36											

similar effect was also observed in *Fagus sylvatica* (Hagedorn et al. 2016). Hagedorn et al. (2016) showed that European beech also increased  $A_n$  and C allocation to ECM (C-sink) during recovery after drought. They concluded that C allocation is sink-driven because an initial excess of C signal in ECM was observed, followed by a delayed excess of photosynthetic activity, according to the hypotheses on source–sink control tested here. In the present experiment, we obtained similar results: reduced irrigation decreased  $A_n$ , rewatering led to recovery but not an excess of  $A_n$ , while an excess allocation of recent photosynthates was observed in ECM even though drought reduced the relative number of below-ground sinks. This indicates sink driven C allocation. We additionally tested different nutrient levels in the soil and found significantly higher C allocation to ECM in recovering plants of non-N-fertilised treatments. This C is most probably used to restore roots and functions of ECM (i.e. uptake of water and nutrients) after drought. Effects on root growth are unlikely

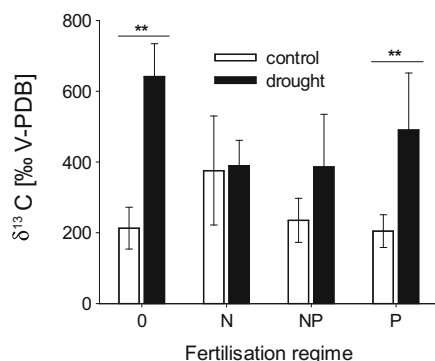
as increased root growth after rewatering would have resulted in a relative decrease of mycorrhization—which was not observed here—because ECM formation takes longer than one week. The same trend was observed in N-fertilised treatments, but the effect was not significant. By implication, under high N availability, there might be less need to restore root and ectomycorrhizal metabolism as N can be used to synthesise amino acids, many of which—especially proline—can also act as osmoprotectants. These molecules accumulate during water shortage not inhibiting cellular metabolism, but protecting cells from damage (Handa et al. 1986; McNeil et al. 1999; Rhodes et al. 1986; Yancey et al. 1982). In a microcosm experiment, Bidartondo et al. (2001) showed that N uptake increased the C sink strength of ectomycorrhizal fungi due to the high C and energy demands. However, in sufficiently irrigated treatments, C allocation was not significantly higher when N fertiliser had been applied. Therefore, we suggest that, despite the high energy demand of N uptake,



**Fig. 3** **a)** Net photosynthesis ( $A_n$ ) and **b)** instantaneous water use efficiency (WUE) of *P. maximowiczii* × *nigra* clone ‘Rochester’ during the experiment in irrigated (open circles) and drought-stressed plants (open triangles) separated by fertilisation regime (+0 = no fertilisation, +N = N fertilisation, +P = P fertilisation, +N + P = N and P fertilisation). Curves show LOESS fits with the 95% confidence interval (grey shading) of irrigated (solid line) and drought-stressed (dashed line) plants. Data obtained from steady-state gas exchange measurements that were performed on leaves 9–11 (from apex) measured under average growth conditions (25 °C and 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD). Shading and numbers above the x-axis indicate water supply of drought treatments relative to continuously irrigated treatments

the better nutritional status of the N-fertilised plants during recovery lowered the impact of stress and thus the C sink strength of ECM. This is also emphasised by the overall significantly higher C allocation to the ECM of recovering plants, when no P was applied as fertiliser. An example for stress induced C allocation is, that the sink strength of poplar leaves was higher after insect wounding and the application of jasmonic acid (Arnold and Schultz 2002). The fertilisation regime had no significant influence on C allocation to ECM under continuously watered conditions. The added N was taken up before harvest under irrigated conditions, as indicated by the results of soil mixture N content and NBI measurements. Therefore, a direct effect of fertilisation might not have been observable.

In our experiment, poplar plants showed increased instantaneous WUE under drought, which was sustained after rewatering in the plants without N fertiliser, similar to the findings of Liu et al. (2005) in *Glycine max* under laboratory conditions. As expected,

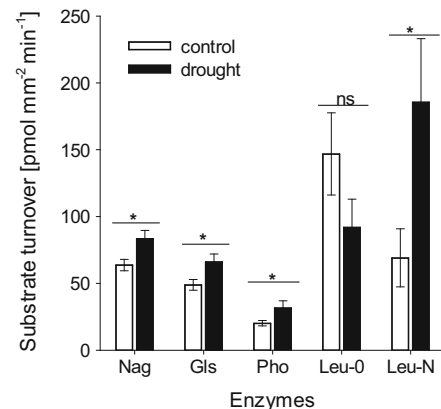


**Fig. 4**  $\delta^{13}\text{C}$  values of ECM tips of *P. maximowiczii* × *nigra* clone ‘Rochester’ at harvest, from continuously watered plants compared with drought-stressed plants one week after rewatering, one day after labelling. 0 = no fertilisation, N = nitrogen fertilisation, NP = nitrogen and phosphorus fertilisation, P = phosphorus fertilisation. \*\* =  $p < 0.005$

**Table 6** Analysis of covariance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on  $^{13}\text{C}$  abundance in ECM of *P. maximowiczii* × *nigra* clone ‘Rochester’ one day after labelling. Leaf area was used as a covariate to correct for different assimilation areas of the plants. Significant results ( $p < 0.05$ ) are in bold

Source of variation	$^{13}\text{C}$ abundance in mycorrhizae		
	df	F	p
Leaf area	1	0.25	0.618
Irrigation	1	8.07	<b>0.008</b>
N-fert.	1	5.27	<b>0.028</b>
P fert.	1	0.43	0.518
Irrig x N	1	0.71	0.404
Irrig x P	1	4.68	<b>0.038</b>
N x P	1	0.03	0.854
Irrig x N x P	1	0.09	0.766
Residual	33		

drought reduced growth while nitrogen fertilisation enhanced it. Similar morphological responses of *Populus* species to drought stress have been reported in previous experiments (Ibrahim et al. 1997; Yin et al. 2005; Zhang et al. 2004). Higher above-ground growth is also a higher above-ground C sink, which could explain the lower  $^{13}\text{C}$  abundance in ECM of recovering plants that received N fertiliser alternatively to the better nutritional



**Fig. 5** Enzymatic activities of mycorrhizal root tips of *P. maximowiczii* × *nigra* clone ‘Rochester’ at harvest, from continuously watered plants compared with drought-stressed plants one week after rewatering. Nag = chitinase, Glu =  $\beta$ -glucosidase, Pho = phosphatase, Leu-0 = leucine aminopeptidase not N-fertilised plants, Leu-N = leucine aminopeptidase N-fertilised plants. Nag, Glu and Pho activities are significantly higher in ECM tips of plants recovering from drought. There is a significant interaction effect of irrigation regime and nitrogen fertilisation on Leu activity. \* =  $p < 0.05$



**Table 7** Mean values and standard errors ( $n = 4-6$ ) of exoenzyme activities (pmol  $\text{mm}^{-2} \text{min}^{-1}$ ) of ECM tips of *P. maximowiczii*  $\times$  nigra clone 'Rochester' at harvest, one week after rewatering. The treatments are irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Below: Analysis of variance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on selected exoenzyme activities of mycorrhizae of *P. maximowiczii*  $\times$  nigra clone 'Rochester'. Significant results ( $p < 0.05$ ) are in bold

Treatment	Leucine-aminopeptidase		Xylosidase		Glucuronidase		Cellobio-hydrolase		Chitinase		$\beta$ -1,4-glucosidase		phosphatase			
	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se		
C0	115.98	23.84	31.62	5.26	17.94	7.63	26.73	6.86	65.84	9.15	45.71	7.21	21.60	4.52		
CN	72.93	34.00	35.56	7.22	13.96	6.96	23.59	6.45	62.18	7.46	41.34	7.38	17.94	2.98		
CNP	63.36	24.95	44.12	16.64	19.98	8.42	28.91	9.62	64.99	12.73	52.86	9.02	23.46	6.81		
CP	177.74	56.60	50.74	8.32	15.51	7.94	26.21	8.88	62.37	8.65	57.00	8.98	19.00	3.01		
T0	108.40	31.64	42.35	11.14	12.82	2.10	25.17	2.47	62.12	3.00	56.30	5.51	21.08	1.09		
TN	226.41	74.05	59.80	17.63	21.42	4.71	34.69	2.85	97.93	7.03	84.96	11.14	37.87	5.84		
TNP	124.27	33.96	42.66	15.64	18.73	5.98	32.44	9.40	91.51	21.35	64.52	16.87	24.61	3.75		
TP	75.50	29.09	45.60	19.36	35.46	20.22	52.81	21.31	85.01	14.44	58.29	12.60	40.74	18.49		
Source of variation	F	p	F	p	F	p	F	p	F	p	F	p	F	p		
Irrigation	1	0.41	4E-04	0.985	0.77	0.386	3.05	0.089	7.24	<b>0.011</b>	4.49	<b>0.041</b>	6.41	<b>0.016</b>		
N-fert.	1	0.09	0.12	0.733	0.34	0.562	0.01	0.904	1.70	0.201	0.34	0.563	0.33	0.571		
P fert.	1	0.31	0.14	0.712	0.01	0.906	0.13	0.725	0.08	0.778	0.02	0.881	0.01	0.928		
Irrig x N	1	6.68	<b>0.014</b>	1.36	0.251	0.33	0.566	0.04	2.00	0.166	1.93	0.173	0.63	0.432		
Irrig x P	1	1.26	0.270	1.32	0.258	0.16	0.690	0.07	0.25	0.623	2.64	0.113	0.01	0.916		
N x P	1	0.25	0.617	0.33	0.571	0.38	0.540	0.17	0.76	0.390	0.24	0.626	0.50	0.486		
Irrig x N x P	1	0.11	0.739	0.28	0.597	0.19	0.666	1.21	1.46	0.235	0.45	0.508	2.72	0.108		
Residual	36															

status of the plants. Yet, this would underpin the hypothesis of source-driven allocation of C. However,  $^{13}\text{C}$  abundance in leaves of recovering N-fertilised plants was not higher than in not fertilised plants, but this may result from dilution by assimilation of ambient  $\text{CO}_2$  as plants were harvested one day after labelling. Thus, we cannot finally clarify if the lower  $^{13}\text{C}$  abundance in ECM tips of recovering N-fertilised plants is due to the better nutritional status of the plants or due to the partitioning of recent photosynthates between two sinks.

More non-vital ECM tips were found in the drought treatments and this share was not influenced by the fertilisation regime. AM colonisation was previously reported to be very low on roots of *Populus tremuloides* in a field experiment (Neville et al. 2002). As the authors found evidence that the occurrences of ECM and AM are negatively correlated and linked to soil depth, the absence of AM in our experiment may partly be due to a pot effect.

We found no support for our hypothesis that mineral fertilisation alone reduces the enzyme activities of ECM. In fact, leucine aminopeptidase activity was higher in N-fertilised ECM of recovering plants than in continuously irrigated plants. This is an intriguing result as proteolytic activity of the ectomycorrhizal fungus *Hebeloma crustuliniforme* has been shown to be reduced in the presence of easily available N (Zhu et al. 1994). Another explanation could be higher N demand by N-fertilised plants due to their larger size and because the N content was very low in all treatments at harvest. Our hypothesis that the enzyme activities of ECM increase upon recovery from drought was confirmed by three out of seven enzymes, whereas the activities of the other four enzymes did not respond significantly to the drought treatment. Exoenzyme activities of ECM can be very stable upon disturbance. It has been shown that ectomycorrhizal communities can maintain the level of their enzymatic activities under stress conditions (Diedhiou et al. 2010; Jones et al. 2010). Even within a single ectomycorrhizal species leucine aminopeptidase activity was regulated depending on the abundance of this species to maintain a stable activity (Herzog et al. 2013). Herzog et al. (2013) found neutral responses to drought in the activities of the same seven enzymes we measured in *Cenococcum geophilum* ectomycorrhizae of different *Quercus* species. The authors attributed this result to the generalist lifestyle of *C. geophilum* in contrast to specialist species as for example *Lactarius subdulcis* or *Xerocomus pruinatus*, because these

specialist species accounted for most of the effects of liming on the EA profile of an ectomycorrhizal community in a Norway spruce and beech stand (Rineau and Garbaye 2009). In our experiment the dominant ectomycorrhizal species *S. brunnea* is also most probably a generalist as suggested by its wide distribution and host range (Danielson 1984), but with upregulation of activities of three out of seven enzymes which underlines the significance of these alterations. Higher activities of  $\beta$ -glucosidase and chitinase in ECM of recovering plants suggest the degradation of plant and fungal cell walls and arthropod-derived chitin from individuals that died during drought (Pritsch and Garbaye 2011). This supports the need to mobilise N, as reflected in the similar increase in leucine aminopeptidase activity. A high proportion of dead roots and ECM may have stimulated cellulolytic and chitinolytic activities in the remaining vital ECM in order to attain N- and P-rich substrates inside these dead cells and tissues. Furthermore, the uptake of  $\text{NH}_4^+$  (and  $\text{NO}_3^-$ , which is eventually metabolised to  $\text{NH}_4^+$ ) requires C to form the C skeleton for glutamate or glutamine. Although this C is supplied to the fungus by the plant, the breakdown of proteins and enhanced  $\beta$ -glucosidase activity may augment plant C supply (Abuzinadah and Read 1988; Chalot and Brun 1998; Chalot et al. 1994), as up to 40% of the supplied C has been shown to be used for N assimilation in culture experiments with ectomycorrhizal fungi (Martin and Canet 1986; Martin et al. 1988). Enhanced phosphatase activity during recovery from drought may help to mobilise P from dead cells and tissues, which were made accessible by the chitinolytic and cellulolytic activities of ECM. After severe drought, there might also be a need for phosphorus to restore metabolism.

The utilisation of supplied N was much higher in irrigated plants than in drought-treated ones, as shown by their enhanced growth and the rapid change in NBI after fertilisation. However, leaf N content at harvest was higher in recovering plants, probably reflecting retarded N uptake during the early phase of recovery and because low water availability in the rhizosphere may have decreased ion uptake and transport to shoots (Greenway et al. 1969; Pitman 1981). N enrichment may thus be a result of reduced absorption processes in the root and ion movement in the soil during drought (Dunham and Nye 1976; Russell 1973). Even N-fertilised plants were N-limited at harvest as suggested by leaf NBI development and N:P ratios ranging from

ca. 1.5 to 5.5 which is below the optimal value of 9 for *P. nigra* × *P. maximowiczii* (Kelly and Ericsson 2003). The fertiliser design mirrored agricultural practice with one fertilisation application at the beginning of the experiment in contrast to continuous fertilisation. As the soil mixture was entirely rooted at harvest, N-supply calculated by pot surface might have been too low considering the pot volume. Yet, we found strong N-fertilisation effects on C allocation to ECM and—depending on the irrigation regime—on measures of growth. Plants of non-P-fertilised treatments might not have experienced P limitation as reflected by plant growth and leaf P content (Jug et al. 1999; van den Burg 1985). For example, field crops did not show P deficiency, despite reduced fertilisation for 5 years, and no adaptation of the soil microbial community was observable (Browne et al. 2009; Conry and Hogan 2001). In fact, P fertilisation in our experiment resulted in slight growth suppression, which was counteracted by N fertilisation, as shown by the significant N × P interaction for leaf biomass and the same trend for shoot biomass. This indicates slight P toxicity because of the high amounts of P added at the beginning of the experiment. This amount was calculated according to guidelines for farmland. We added the missing amount of P for a rich supply. However, the amount of P present in the substrate before fertilisation might have been underestimated because only plant available P was measured. Probably there was labile P in the substrate which was made available by soil biota during growth of the plant (Frossard et al. 2000). In *Eucalyptus grandis*, a suppressive effect of high, spatially homogeneous P fertilisation on shoot growth has also been reported and attributed to reduced nitrate uptake at high P levels (Costa et al. 2016; Graciano et al. 2009). Trends in <sup>15</sup>N contents of leaves of N-fertilised plants in our experiment support these findings, although not significantly.

## Conclusion

Our results indicate that allocation of recent photosynthates is most likely sink-controlled because, after drought release, source activity ( $A_n$ ) recovered to the level in irrigated plants, but <sup>13</sup>C allocation to ECM (C-sink) was much higher. However, this was less pronounced in the presence of N and P fertiliser. Overall, this suggests that either (1) sink strength is an indicator of the impact of drought stress and that this sink strength

can be reduced especially by fertilisation with N, or (2) that recent photosynthates were partitioned between two sinks (i.e. ECM and above-ground growth). Testing a wider range of nutrient stoichiometries and quantifying above- and below-ground sink strength would reward further studies investigating the interplay of carbon source-sink dynamics in recovering plants and nutrient availability in soils.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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