Phosphorus stress strongly reduced plant physiological activity, but only temporarily, in a mesocosm experiment with *Zea mays* colonized by arbuscular mycorrhizal fungi

Melanie S. Verlinden[1], Hamada AbdElgawad[2,3], Arne Ven[1], Lore T. Verryckt[1], Sebastian Wieneke[1,4], Ivan A. Janssens[1], Sara Vicca[1]

[1]Plant and Vegetation Ecology (PLECO), Department of Biology, University of Antwerp, Wilrijk, 2610, Belgium

[2]Botany and Microbiology Department, Faculty of Science, Beni–Suef University, Beni–Suef, 62521, Egypt

[3]Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, Antwerp, 2020, Belgium

[4]Remote Sensing Centre for Earth System Research (RSC4Earth, Faculty of Physics and Earth Sciences, University of Leipzig, Leipzig, 04109, Germany

Correspondence to: Melanie S. Verlinden (Melanie.Verlinden@uantwerpen.be); Sara Vicca (Sara.Vicca@uantwerpen.be)

Abstract. ~~Despite being~~ Phosphorus (P) is an essential macronutrient for plant growth~~, phosphorus (P) is~~ and one of the least available nutrients in ~~soils and~~soil. P limitation is often a major constraint for plant growth globally. Although P addition experiments have been carried out to study the long-term effects on ~~the~~ yield, data on P addition effects ~~to~~on seasonal variation ~~in~~of leaf-level photosynthesis are scarce. Arbuscular mycorrhizal fungi (AMF) can be of major importance for plant nutrient uptake, and AMF growth may be important for explaining temporal patterns in leaf physiology. In a nitrogen (N) and P fertilization experiment with *Zea mays*, we investigated the effect of P limitation on leaf pigments and leaf enzymes, how these relate to leaf-level photosynthesis, and how these relationships change during the growing season. Previous research indicated that N addition did not affect plant growth and also the leaf measurements in the current study were unaffected by N addition. Contrary to N addition, P addition strongly influenced plant growth and leaf-level measurements. At low soil P availability, leaf-level photosynthetic and respiratory activity were strongly decreased and this was associated with reduced chlorophyll and photosynthetic enzymes. Contrary to the expected increase in P stress over time following gradual soil P depletion, plant P-limitation decreased over time. For most leaf-level processes, pigments and enzymes under study, the fertilization effect had even disappeared two months after planting. Our results point towards a key role for the AMF-symbiosis and consequent increase of P uptake in explaining the vanishing P stress.

1 Introduction

Phosphorus (P) is ~~an essential macronutrient for plant growth, playing a role in most developmental and biochemical processes in plants. Structurally, P participates in the formation~~a crucial element in natural ecosystems. It is present in the structure of DNA, in cell membranes, in molecules storing and supplying energy and in several enzymes. As a consequence, P plays a crucial role in plant and soil processes, it regulates productivity and ecosystem functions and influences organisms from the individual to the community level (Elser

et al., 2000; Vitousek et al., 2010; Peñuelas et al., 2013). The importance of P for the functioning of the Earth's biogeochemical cycles, especially the carbon cycle, is therefore being increasingly recognized (Vitousek et al., 2010; Wieder et al., 2015; Vicca et al., 2018) and this is reflected in the recent efforts to include P in terrestrial biosphere models (Wang et al., 2010; Gong et al., 2012; Thum et al., 2019).

In plants, P plays a role in most developmental and biochemical processes. Structurally, P is a component of RNA and membrane phospholipids, while metabolically, P functions in the storage and transfer of energy and in energizing of binding sites for metabolic turnover (Schulze et al., 2005~~).~~.; Veneklaas et al., 2012). However, P is one of the least available macronutrients in soils, and P limitation is often a major constraint for plant growth (Augusto et al., 2017). ~~More~~On more than one third of the arable land worldwide, plant productivity is considered to ~~experience~~be limited by P ~~stress~~ (Calderón-Vázquez et al., 2009).

Various experiments have been conducted to study the effect of P addition to crops, thereby mainly focusing on the long-term effect on ~~the~~ yield (Khan et al., 2018; Johnston and Poulton, 2019). However, data on seasonal variation in leaf-level photosynthesis, especially in crops, are scarce (Rodríguez et al., 2000; Rogers, 2014), while accurate seasonal estimates of photosynthetic capacity are critical for modelling the time course of carbon fluxes (Miner and Bauerle, 2019). The majority of studies investigating effects of nutrients on photosynthesis focus on nitrogen (N) and much less on P and other nutrients~~. In addition~~ (e.g., Brooks, 1986; Brooks et al. 1988; Rodríguez and Goudriaan, 1995; Rodríguez et al., 1998). In addition, it is unclear whether leaf traits, such as leaf nutrients, pigments and enzymes, change seasonally in relation to leaf-level photosynthesis.

Among others, plant P limitation typically results in reduced photosynthesis and plant growth, especially aboveground. P is required for adenosine triphosphate (ATP) synthesis~~,~~ (Veneklaas et al., 2012), which is needed to regenerate Ribulose 1,5-bisphosphate (RuBP) in the Calvin cycle of photosynthesis. Inorganic phosphate (Pi) directly affects the activity of Calvin cycle enzymes through the level of activation. For instance, Pi is required for light activation of Rubisco (Parry et al., 2008). It also directly affects maximum rate of CO_2-limited carboxylation (v_{cmax}) and triose phosphate utilization (Lewis et al., 1994) and RuBP-regeneration-limited rates of electron transport (Loustau et al., 1999). P-deficiency therefore leads to a decrease in RuBP pool size and insufficient ATP, and consequently to a decrease in photosynthetic C assimilation. The ~~content~~concentration and specific activity of ~~rubisco~~Rubisco, the primary CO_2 fixing enzyme in photosynthesis, are generally little affected by P~~-~~ stress (Brooks, 1986; Paul and Stitt, 1993; Pieters et al., 2001, but see Jacob and Lawlor, 1991; Pieters et al., 2001). ~~Leaf starch, playing an important role in the cellular recycling of phosphate for~~

Pi can also indirectly affect photosynthesis~~, often increases~~ through the changes in stromal pH (Bhagwat, 1981), where the consumption of Pi as a substrate of photosynthesis could decrease photosynthesis by a direct effect of low stromal Pi concentration on Rubisco. Moreover, the effect of P on photosynthesis depends on the dynamic interactions between sink and source tissues. Low P can reduce carbon export to sinks, and thus decrease sink strength, thereby limiting photosynthesis (Pieters et al., 2001). Concomitantly, leaf starch can increase with P~~-~~ stress (Zhang et al., 2014) due to low availability of P for triphosphate translocation, ~~however~~although decreases of leaf starch have also been observed (Halsted and Lynch, 1996). Moreover, low sink strength restricts the recycling of Pi back to the chloroplast, further reducing photosynthesis (Paul and Foyer, 2001).

In a mesocosm nutrient manipulation experiment setup (previously described in Verlinden et al., 2018), maize (*Zea mays* L.) was planted at different soil N and P availabilities. As demonstrated in Verlinden et al. (2018), this resulted in a strong P, but no N effect on plant growth or photosynthesis at mesocosm scale. In that study, also

arbuscular mycorrhizal fungi (AMF) played an important role in explaining plant carbon uptake and allocation. AMF are important for nutrient uptake in maize (Hartnett and Wilson, 1999; Hoeksema et al., 2010), especially for P, and hence AMF growth may also be important for explaining variation in leaf physiology. The objective of the current study is to ~~verify~~test the effect of P limitation on leaf pigments, sugars and ~~leaf~~photosynthetic enzymes, how they relate to leaf-level photosynthesis, and how these relationships change during the growing season. At low soil P availability, we expected low leaf-level photosynthetic and respiratory activity, associated with reduced chlorophyll and photosynthetic enzymes. Furthermore, P-stress was expected to increase over time, as plants were expected to gradually deplete the soil P.

2 Material and methods

2.1 Experimental design

~~A mesocosm experiment consisting~~For this study, we used the first of two mesocosm fertilization experiments. While the first applied a full-factorial NxP fertilization approach and was first described in Verlinden et al. (2018), the second applied a P gradient. Results for the latter are reported in Ven et al. (2020b). The mesocosm experiment consisted of 20 (1 m × 1.2 m, 0.6 m high) insulated boxes was set up in a greenhouse in Sint-Katelijne-Waver, Belgium (51°04'38" N, 4°32'05" E). To each mesocosm we added soil, which was a homogenized mixture of sand originating from a pine forest in a nature reserve in Flanders, white river sand and a minority of compost (details of the experimental setup are described in Verlinden et al. (2018)). On 20 May 2016, 12 seedlings of maize (*Zea mays* L., variety 'Tom Thumb') were planted per mesocosm. Different treatments (set up in five replicates) were distinguished in the level of nutrients added: the +N treatment was fertilized with calcium nitrate at a rate of 95.5 kg N ha^{-1} (YaraLiva® Calcinit®), the +P treatment received 20 kg P ha^{-1} as triple superphosphate (Janssens-Smeets®), the combined +N and +P treatment (+NP) received both amounts together. The control treatment received, as all other treatments, only a basic level of micronutrients (Fertigreen® Patentkali® and GroGreen® containing in kg ha^{-1}: 79 Potassium, 19 Magnesium, 53 Sulfur, 0.4 Boron, 0.1 Copper, 2.4 Iron, 1.1 Manganese, 0.1 Molybdenum, 0.4 Zinc). Spores-based inoculum of AMF (species *Rhizophagus irregularis*, Symplanta®) was added to all 20 (4 treatments × 5 replicates) mesocosms. Soil moisture was monitored and kept at a non-limiting (field capacity) level, similar in all plots.

2.2 Measurements and analyses

2.2.1 Leaf C, N and P concentration and Specific Leaf Area

Carbon (C) and N concentrations were determined using an elemental analyzer - model FLASH 2000 (Thermo Fisher Scientific, Waltham, USA). Total leaf P concentration was determined by digestion in tubes with H_2SO_4-salicylic acid- H_2O_2 and selenium (Temminghoff and Houba, 2004). Specific Leaf Area (SLA; $m^2\ kg^{-1}$) was determined as the ratio of the fresh leaf area and dry leaf mass.

2.2.2 Leaf Photosynthesis

A portable gas exchange system LI-6400 (LI-COR, Lincoln, NE, USA) was used for leaf scale CO_2 gas exchange measurements, operating as an open system (e.g. Verlinden et al., 2013). Leaf-scale measurements were performed

during two weeks late June (campaign 1, C1) and repeated end of July (campaign 2, C2), allowing to study the seasonal ~~evolution~~development. Mean daily photosynthetically active radiation (PAR) during C1 and C2 were respectively 17.1 and 17.7 mol·m^{-2} and average temperature respectively 21.7 and 23.3 °C.

In each plot photosynthetic CO_2-response curves (i.e. photosynthesis (A, assimilation) responses to the CO_2 concentration inside leaf air spaces (c_i)) were measured on a recently matured leaf. Leaves were allowed to equilibrate at a CO_2 concentration of 400 µmol mol^{-1} in the leaf cuvette, after which the net CO_2 assimilation rate at a sequence of different CO_2 concentrations (i.e. 400, 30, 50, 80, 110, 150, 250, 350, 500 and 1000 µmol mol^{-1}) was measured. Photosynthetic photon flux density (PPFD) was fixed at a saturating value of 1200 µmol s^{-1} m^{-2}. The resulting A-c_i data were fitted to the biochemical model of C_4 photosynthesis as presented by von Caemmerer (2000) using the package 'Plantecophys' (Duursma, 2015) in R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). The CO_2 assimilation rate is approximated by the minimum of the expressions of an enzyme-limited and an electron-transport-limited CO_2 assimilation rate. The parameters J_{max} (maximum electron transport rate), v_{cmax} (maximal rubisco carboxylation rate) and v_{pmax} (maximum PEP carboxylation rate) were calculated through curve fitting based on minimum least-squares.

Photosynthetic 'light- response ~~curves~~curves' were obtained by measurements of the net CO_2 assimilation rate at PPFD's of 1200, 500, 250, 100, 80, 60, 40, 30, 25, 20, 15, 10, 5 and 0 µmol m^{-2} s^{-1} (blue-red LED source type 6400-02B, 13% blue light). Leaves were allowed to equilibrate at each step before logging the data. The CO_2 concentration in the cuvette was maintained at 400 µmol mol^{-1} and the block temperature at 25°C. From the 'light response ~~curves~~curves', the net CO_2 assimilation rate at light saturation (A_{max}) and leaf dark respiration (R_{dark}, net CO_2 exchange at zero light) were derived. In addition, light-induced inhibition of leaf respiration was estimated from the 'light response ~~curves~~curves' (for PPFD's 0 to 80 µmol m^{-2} s^{-1}) from the intersections of the fitted lines above and below the light compensation point the y–axis, giving respectively R_{light} and R_{dark} (Kok, 1948). All selected leaves were harvested and stored at -80°C for later analyses.

2.2.3 Chemical analyses of leaf material

Rubisco activity was analyzed according to Sulpice et al. (2007). It was expressed as the conversion rate of ~~phosphor~~ glycerate kinase (3-PGA) of extracted leaf samples, in µmol 3-PGA m^{-2} min^{-1}. The activity of Rubisco was determined directly ('direct rubisco'), without incubation of the extract in the presence of 10 mM HCO^{3-} and 20 mM Mg^{2+} to convert the non-carbamylated Rubisco into the carbamylated form. The assay of phosphoenolpyruvate carboxylase (PEPC) was coupled with the malate dehydrogenase reaction, the resulting ~~measure~~rate of PEPC activity was expressed in µmol HCO_3 m^{-2} min^{-1}.

Mono- and oligosaccharides in ~~leaf tissue~~leaves were analyzed chromatographically according to AbdElgawad et al. (2014). Soluble sugar concentrations were measured by high performance anion exchange chromatography of extracted leaf samples with pulsed amperometric detection (HPAEC-PAD) and the total soluble ~~sugars~~sugar concentration was calculated as their sum. The remaining pellet of soluble sugars extraction was treated with a mixture of α-amylase and amyloglucosidase to extract starch.

High-performance liquid chromatography (HPLC) was used to analyze leaf pigments. The detection of the carotenoids and xanthophylls was done by a diode array detector (Shimadzu SPD-M10Avp, Kyoto, Japan) at four ~~different~~ wavelengths (420, 440, 462, 660 nm) and integrated via the software program (Shimadzu Lab Solutions Lite, Kyoto, Japan) in which the concentration was determined using a calibration curve.

2.2.4 Mycorrhizal fungi

Because AMF growth is potentially crucial for explaining patterns in the leaf response to P limitation, we determined the time course of AMF abundance in each of the mesocosms. To this end, five mesh bags filled with white river sand – and permeable for fungi but not for roots (30 µm mesh size) – were buried vertically into the top soil of each mesocosm one week before planting. They were harvested consecutively 31 (corresponding to C1, *v.i.*) and 61 days (right before C2) after planting. Hyphae were extracted from 4 g mesh bag sand using the method of Rillig et al. (1999). After suspending, processing and ~~colouring~~staining the sample, hyphal intersects were counted at a magnification of 40 × 10 using a grid in the microscope ocular. Hyphal length density was calculated following Eq. (1) (Tennant, 1975; Rillig et al., 1999):

$$HLD = (\pi \cdot n \cdot a \cdot d) \cdot (h \cdot w)^{-1}, \quad (1)$$

where HLD = hyphal length density (mm hyphae g^{-1} soil), n = number of intersects containing AMF hyphae, a = filter area (mm^2) examined, d = dilution factor, h = total length of raster lines projected on filter (mm), and w = soil weight (g).

Mycorrhizal colonization was examined in C1 and C2 by sampling roots from two plants per mesocosm. Per plant, 20 cm of one lateral root containing root hair, was excavated, cut, and stored. Mycorrhizal colonization was quantified by counting arbuscules, vesicules, and hyphae applying the gridline intersection method (Vierheilig et al., 2005). The methodology on root colonization determination is described more elaborately in Verlinden et al. (2018).

2.2.5 Statistical Analyses

Data normality and homoscedasticity were checked using the Shapiro-Wilk and Levene's test, respectively. A three-way mixed analyses of variance (ANOVA) was applied to test if the quantified variables differed between the treatments and between C1 and C2. N addition and P addition were both considered as between-subject variables and time (campaign) as a within-subject variable. Non-significant interactions terms, and further, non-significant factors were removed from the model. In case of significant interaction between factors, the analysis included their multiplied factor levels. A Tukey post-hoc test was applied for pairwise comparison in case of significant factor effects.

3 Results

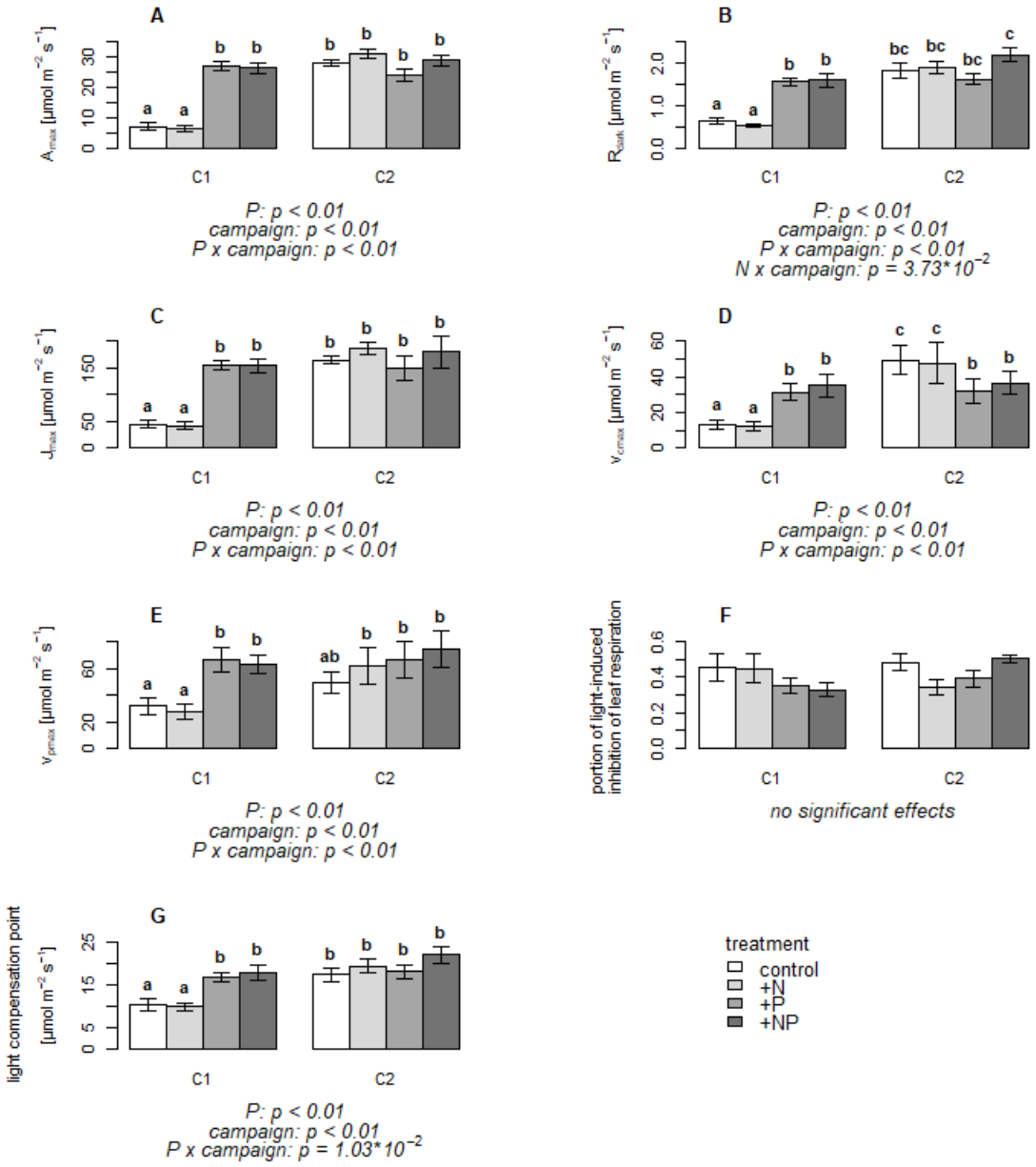
The addition of P-fertilizer increased soil P availability (Verlinden et al., 2018), as well as leaf P concentration (Table 1). At the time of C1, leaf P concentration was three to four times higher in the +P and +NP treatments than in the non-P-fertilized control and +N treatments. Leaf N:P ratio was higher in the non-P-fertilized treatments than in the P-fertilized treatments (an average N:P ratio of 19.8 versus 37.2 for the non-P-fertilized treatments). However, in C2, the leaf P concentration had increased in all treatments to a similar level (Table 1), as well as the N:P ratio, which decreased for all treatments to a similar level with a mean of 13.8. Leaves in the non-P-fertilized mesocosms were thinner ~~as compared to~~and/or had a lower density than in the P-fertilized mesocosms (Table 1) during C1, with mean SLA values of respectively 52.9 ± 0.9 and 33.9 ± 1.9 $m^2\ kg^{-1}$. Towards later in the season, SLA decreased in all mesocosms and the difference between non-P-fertilized and P-fertilized mesocosms had disappeared at the time of C2.

The majority of leaf physiology parameters differed considerably between C1 versus C2 for the non-P-fertilized treatments, while for the P-fertilized treatments differences between C1 and C2 were much less pronounced. During C1, photosynthetic activity was very low in the non-P-fertilized treatments, with a mean A_{max} of 6.2 (± 4.1) µmol m^{-2} s^{-1} for the control and +N treatments. In contrast, the +P and +NP treatments showed a mean A_{max} more than four times higher than in the non-P-fertilized mesocosms (Fig. 1A). A similarly high A_{max}-level was reached for all treatments in C2 (Fig. 1A). Also R_{dark} was smaller in the non-P-fertilized treatments in C1 (Fig. 1B) and reached a similar level as the +P and +NP treatments in C2. Photosynthetic parameters J_{max}, v_{cmax} and v_{pmax} were all lower in the non-P-fertilized treatments than in the P-fertilized treatments during C1 (Figs. 1C-E), but by the time of C2, J_{max} had increased in the non-P-fertilized mesocosms to the level of the P-fertilized mesocosms. V_{cmax} in the non-P-fertilized mesocosms had even increased to a level of about 45% higher than the P-fertilized mesocosms, while the P-fertilized mesocosms showed very similar J_{max}, v_{cmax} and v_{pmax} for C1 and C2. Light-induced inhibition of respiration (Fig. 1F) was variable amongst the mesocosms, though on average it tended to be higher in the non-P-fertilized mesocosms during C1, whereas no trend was observed during C2. The light compensation point was initially lower in the non-P-fertilized plants (i.e., in the stressed plants photosynthetic activity occurred at a lower light availability than in the P-fertilized treatments), whereas during C2 no differences were observed between the mesocosms (Fig. 1G).

Similar to the gas exchange measurements, the leaf chemistry showed a strong difference between non-P-fertilized and P-fertilized plots during C1, but not during C2. Direct rubisco concentration was initially lower in the non-P-fertilized mesocosms (Table 1), which was also true for the enzyme PEP-carboxylase (Table 1). A P- and campaign effect was observed for total chlorophyll (Table 1, similarly for $chlorophyll_a$ and $chlorophyll_b$, data not shown), its concentration was four times higher in the P-fertilized mesocosms during C1. Also beta-carotene concentration was initially higher in the ~~non-~~P-fertilized mesocosms (Table 1). Zeaxanthin was only detected in the non-P-fertilized leaves during C1 (Table 1). For both lutein and violaxanthin no differences among the treatments were observed during C1. There was a tendency of lower starch in the P-stressed mesocosms as compared to the P-fertilized mesocosms during C1 although there was no P effect, whereas the campaign effect and interactions P x campaign and N x P x campaign were significant.

During C2, direct rubisco concentration increased in the non-P-fertilized mesocosms to the same level as in P-fertilized mesocosms, while PEP-carboxylase concentration increased in all mesocosms to reach a similar level in C2. Chlorophyll concentration increased more than 12 times for the non-P-fertilized mesocosms from C1 to C2; for the P-fertilized mesocosms almost four times. A similar trend was observed for beta-carotene (Table 1), of which concentrations increased five- and threefold respectively. Also lutein and violaxanthin were present in higher concentrations during C2 (Table 1). Zeaxanthin was not detected during C2. The leaf starch concentration differed over time, leaves contained much less starch during C1 ~~compared to~~than during C2 (Table 1).

One month after establishing the experimental setup (during C1), no AMF were detected in plant roots or in the meshbags (Fig. 2). One month later, i.e. during C2, however, AMF had clearly established, with a mean hyphal length density of 760 mm per gram of soil in all treatments. The percentage of roots colonized was higher in the non-P-fertilized treatments than in the P-fertilized plots (67% vs. 40% on average) (Fig. 2; Ven et al., 2020a).



230 **Figure 1 A-G:** Means of parameters deduced from leaf CO_2 exchange measurements per treatment and campaign. Error bars indicate standard error. C1: campaign 1, end of June; C2: campaign 2, end of July; control treatment: not fertilized, +N treatment: nitrogen fertilized, +P treatment: ~~Phosphorus~~phosphorus fertilized, +NP treatment: both nitrogen and phosphorus fertilized. Letters above bars indicate ~~statistical~~significant differences. Significant effects are given with p- value below the plots. A_{max} = maximal assimilation rate; R_{dark} = leaf dark respiration, R_{dark}/A_{max} = ratio of leaf dark respiration to maximal
235 assimilation rate; J_{max} = maximum electron transport rate; v_{cmax} = maximal rubisco carboxylation rate; v_{pmax} = maximum PEP carboxylation rate;

Table 1. Means (and standard errors) of chemically analyzed leaf traits, per campaign and per treatment.

Notes: C1: campaign 1 end of June; C2: campaign 2 end of July. SE: standard error, Superscript letters indicate homogeneous groups as results from post-hoc analysis of statistical analyses of variance.

campaign	treat-ment	leaf N	leaf P	leaf N:P	SLA	direct rubisco	PEP-carboxylase	total chlorophyll	beta-caroteen	zeaxanthin	violaxanthin	lutein	starch	insoluble sugars	soluble sugars
		g m^{-2}	g m^{-2}	-		µmol 3-PGA/m² min	µmol HCO3 /m².min	mg m^{-2}	mg m^{-2}	mg m^{-2}	mg m^{-2}	mg m^{-2}	g m^{-2}	g m^{-2}	mg m^{-2}
p-values:															
P		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	3.36*10^{-2}	< 0.01					
campaign		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
P x campaign		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01			< 0.01		2.78*10^{-2}	< 0.01		
P x N		3.93*10^{-2}			2.25*10^{-2}										
P x N x campaign		4.99*10^{-2}			3.24*10^{-2}								4.22*10^{-2}		
		mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)
C1	control	0.66^{a} (0.03)	50.8^{a} (0.001)	37.1^{a} (1.7)	50.8^{a} (1.5)	39^{a} (8)	66^{a} (4)	82^{a} (41)	33.0^{a} (8.6)	10.3^{a} (3.1)	1.3^{a} (0.8)	6.4^{a} (3.7)	0.33^{a} (0.06)	0.49^{a} (0.03)	52^{a} (3)
	+N	0.66^{a} (0.02)	53.2^{a} (0.001)	37.3^{a} (0.6)	53.2^{a} (0.9)	35^{a} (5)	54^{a} (9)	71^{a} (21)	25.7^{a} (9.2)	7.1^{a} (2.8)	0.3^{a} (0.1)	2.6^{a} (1.3)	0.27^{a} (0.06)	0.45^{a} (0.04)	56^{a} (8)
	+P	1.01^{b} (0.04)	36.4^{b} (0.008)	19.2^{b} (2.0)	36.4^{b} (2.2)	92^{b} (6)	182^{b} (27)	231^{b} (63)	48.9^{b} (3.8)	0^{b} 0	3.5^{a} (1.2)	5.5^{a} (2.6)	0.49^{ab} (0.11)	1.29^{a} (0.13)	73^{a} (12)
	+NP	1.32^{bc} (0.10)	31.3^{bc} (0.008)	20.5^{b} (1.2)	31.3^{bc} (2.9)	112^{b} (6)	218^{b} (21)	351^{b} (79)	52.6^{b} (13.6)	0^{b} 0	4.7^{a} (1.4)	6.5^{a} (2.0)	0.47^{a} (0.06)	1.41^{a} (0.20)	88^{a} (10)
C2	control	1.37^{c} (0.02)	26.4^{cd} (0.003)	13.4^{c} (0.3)	26.4^{cd} (0.7)	95^{b} (9)	275^{c} (28)	944^{c} (41)	137.5^{c} (8.2)	0^{b} 0	20.3^{b} (2.0)	38.2^{ab} (9.0)	1.08^{bc} (0.09)	3.89^{b} (0.63)	212^{b} (15)
	+N	1.49^{c} (0.32)	25.8^{cd} (0.022)	14.5^{c} (0.5)	25.8^{cd} (5.2)	98^{b} (22)	290^{c} (68)	995^{c} (21)	139.0^{c} (30.1)	0^{b} 0	22.9^{b} (6.5)	39.6^{ab} (11.8)	1.39^{c} (0.36)	2.86^{b} (0.65)	192^{b} (47)
	+P	1.36^{c} (0.07)	20.5^{d} (0.005)	14.8^{c} (0.4)	20.5^{d} (1.0)	121^{b} (22)	307^{c} (34)	1119^{d} (63)	153.4^{d} (12.7)	0^{b} 0	28.9^{b} (7.5)	67.4^{b} (6.6)	1.28^{c} (0.21)	3.97^{b} (0.62)	209^{b} (22)
	+NP	1.38^{c} (0.08)	22.5^{d} (0.008)	12.5^{c} (0.7)	22.5^{d} (0.9)	123^{b} (10)	296^{c} (11)	1083^{d} (79)	146.2^{d} (15.7)	0^{b} 0	24.6^{b} (3.1)	63.6^{b} (11.4)	0.86^{abc} (0.06)	3.36^{b} (0.29)	261^{b} (56)

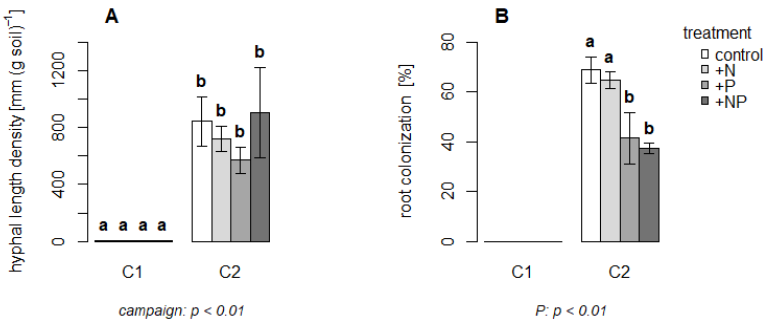


Figure 2: Means of mycorrhizal hyphal length (A) an root colonization (B) by time of C1 and C2. Error bars indicate standard error. Letters above bars indicate statistical differences. ~~Signficant~~Significant effects are given with p-value below the plots

4 Discussion

The unfertilized soil in our experiment ~~can be assumed to be~~was clearly P-~~limited, as previously reported in Verlinden et al. (2018). Addition~~impoverished; addition of P increased plant productivity, whereas N addition did not. End-of-season dry biomass reached 81 (± 7) and 510 (± 24) g m^{-2} for the non-P-fertilized and P-fertilized treatments, respectively (Verlinden et al., 2018). N addition ~~neither~~ had ~~an~~no effect on the leaf-scale measurements, therefore we ~~further~~ focus on effects of P.

Leaf photosynthetic parameters and most leaf chemistry parameters showed clear changes throughout the season, as verified by the significant P x campaign interaction effects (Fig. 1, Table 1). During C1, leaf P concentrations in the non-P-fertilized plants were three times lower than in the P-fertilized plants, whereas leaf P concentrations were similar for non-P-fertilized and P-fertilized treatments during C2. Since growth of plants with leaf N:P ratios higher than 16 (Koerselman and Meuleman, 1996) up to 20 (Güsewell, 2004) ~~are~~is considered to be P-limited, the high leaf N:P ratios of about 37 illustrate a clear P-limitation of plant-growth for the non-P-fertilized treatments in C1, while ~~the~~ P-fertilized treatments ~~are~~were close to P-limitation. In C2, plants ~~seem~~seemed to have reached ~~an optimal~~a favorable allocation of N and P, as shown by the ~~optimal~~favorable N:P ratio (i.e. between 9 and 18, Beauchamp and Hamilton, 1970) ~~similar~~ in all treatments. In accordance, the leaves of the non-P-fertilized plants turned yellow in the first weeks of the experiment, but greened up later.

The initial P-limitation present during C1, strongly limited leaf-level photosynthesis as A_{max}, J_{max} and v_{cmax} were three to four times lower in non-P-fertilized than in P-fertilized plants. This inhibitory effect can be attributed to the decrease in the pool size of ribulose-1,5-bisphosphate (RuBP) and its regeneration (Jacob and Lawlor, 1992; Pieters et al., ~~2001; Calderón-Vázquez et al., 2009). In line with this, rubisco levels were about three times lower in the non-~~2001; Calderón-Vázquez et al., 2009), or by feedback inhibition of photosynthesis, but the latter was not specifically tested. Feedback inhibition of photosynthesis can be induced by elevated soluble sugar levels decreasing the gene expression of photosynthetic enzymes (e.g. PEPC, malic enzyme and RuBisCo) (Jeannette et al., 2000; AbdElgawad et al., 2020). This was not likely the case here, since during C1 sugar levels tended to be lower in the non-P-fertilized than in the P-fertilized treatments. Lower starch and soluble sugar synthesis, like in the non-P-fertilized treatments, can slow Pi regeneration, limit ATP production and eventually the functioning of

the Calvin cycle, which is known as short-term feedback regulation of photosynthesis (Griffin and Seemann, 1996).

Also Rubisco levels were about three times lower in the non-P-fertilized plants than in the P-fertilized plants (Table 1). Insufficient P restricts the conversion of adenosine diphosphate (ADP) to ATP, limiting the RuBP regeneration (Rao and Terry, 1989; Calderón-Vázquez et al., 2009). C_4 plants can maintain adequate levels of P in the bundle ~~sheet~~ cells, and their growth is therefore generally ~~suffer~~ less ~~from~~constrained by P limitation as compared to C_3 plants (Calderón-Vázquez et al., 2009). This ~~implies~~indicates that in our experiment, plants with absent P fertilization must have experienced extreme P limitation early in the season in our experiment. ~~-~~Nonetheless, during C2, photosynthetic parameters reached similar values ~~among~~for all treatments.

Total chlorophyll can drop drastically in case of P deprivation (Jacob and Lawlor, 1991; Usuda and Shimogawara, 1991). In our experiment, chlorophyll concentration was initially lower in the non-P-fertilized mesocosms as compared to the P-fertilized mesocosms. During C2, however, chlorophyll ~~content~~concentration strongly increased in all treatments, both in the initially non-P-fertilized plants where chlorophyll increase was accompanied by increased photosynthesis, ~~but also~~and in the P-fertilized plants. In the latter ones A_{max} did not differ between C1 and C2, indicating that photosynthesis did not increase despite the increase in chlorophyll concentration. Zeaxanthin ~~concentrations were~~was only detected in the non-P-fertilized plants during C1. Schlüter et al. (2013) showed the enhancement of protective pigments, such as zeaxanthin, in maize leaves when growing at low P availability. Zeaxanthin plays a key role in the protection of photosynthetic organisms against excess light, minimizing the over-excitation (Jahns and Holzwarth, 2012; Kuczyńska et al., 2012; Ashraf and Harris, 2013). The xanthophyll violaxanthin is reversibly de-epoxidized to zeaxanthin in the xanthophyll cycle ~~in case~~when the light absorbed exceeds the capacity of photosynthesis. Zeaxanthin synthesis thus acts as a rescuing mechanism in strongly photo-oxidizing conditions (Dall'Osto et al., 2010) and increased zeaxanthin concentrations imply a decrease of light harvesting. In our experiment, no ~~detectable~~ zeaxanthin ~~amounts were~~was detected later in the season, indicating that stress, likely due to P-limitation, ~~seemed~~was relieved and plant growth recovered, as also indicated by the increased net photosynthetic rate.

P deprivation has been found to increase the leaf starch ~~content~~concentration in maize (Zhang et al., 2014), although decreases in starch levels under low P conditions have also been reported (Schlüter et al., 2013). In our experiment, P addition had no clear effect on the leaf starch ~~content~~ concentration (Table 1), ~~but the~~indicating that reduced photosynthetic rates were not due to reduced sink strength. The starch concentration did show a significant campaign effect and more than doubled from C1 to C2. ~~Unlike C_3 plants, synthesis of sucrose and starch in C_4 leaves takes place in different cell types. Whereas starch accumulates in the bundle sheath, sucrose synthesis takes place in the mesophyll (Friso et al., 2010). A shift towards sucrose or starch would thus affect the metabolism of both cell types in different ways.~~ Both sucrose and starch synthesis play important roles in the cellular recycling of phosphate for photosynthesis (Schlüter et al., 2013). A decrease in sugars and starch might lead to lower vitality and productivity of plants, as was previously observed in stressed C_4 leaves (da Silva and Arrabaça, 2004). ~~Due to stress, a larger proportion of starch can possibly be converted to soluble sugars, thereby decreasing the osmotic potential as a form of protection (da Silva and Arrabaça, 2004).~~ In our experiment, while there was no effect of P for both sugars and starch, the campaign effect illustrated an increase of sugars and starch from C1 to C2, possibly suggesting that plants in all treatments experienced nutrient stress during C1. Moreover, the increasing sugar and

starch levels between C1 and C2 further confirm that the low photosynthetic rates for the low P treatments were not due to reduced sink strength.

Foliar respiration rate is suppressed in the light ~~and is known as the 'Kok effect', which is the~~. The abrupt decline in quantum yield of net CO_2 assimilation that occurs at very low light, often near the photosynthetic light compensation point, is also known as the 'Kok effect' (Kok, 1948). This light-induced inhibition of foliar respiration is reported to vary between 25%-100% (see references in Heskel et al., 2013) and is a source of uncertainty in current models of global terrestrial carbon cycling (Heskel and Tang, 2018). It can be impacted by environmental conditions such as temperature and soil nutrient availability (Heskel et al., 2012; Atkin et al., 2013). Here, the light-induced inhibition of respiration was highly variable among measured plants largely ranging from 0.3 to 0.5 with high uncertainty levels. Several studies showed that increased soil nutrient availability can relax the degree of light induced respiration, which was not confirmed in our experiment (Heskel et al., 2012; Atkin et al., 2013; Shapiro et al., 2004).

We applied 20 kg P ha^{-1} for the P treatment at which A_{max} reached its maximum value of about 27 µmol m^{-2} s^{-1}. Zhang et al. (2014) showed that the critical level of P application for maximal net photosynthetic rate of maize (i.e. 30.3 µmol CO_2 m^{-2} s^{-1}) ~~was~~is between 15 and 28 kg ha^{-1}, which is in agreement with our study. Higher P application rates did not result in higher net photosynthetic rates. In our experiment the non-P-fertilized plants reached similar net photosynthetic rates, but only after colonization by AMF during C2. The campaign effect revealed in our experiment, i.e. the remarkable difference in P effect between C1 and C2, was associated with the (slow) establishment of AMF, which may suggest that increased plant P uptake following mycorrhization caused a ~~revival~~recovery of the non-P-fertilized plants, and was beneficial for productivity in the P-fertilized plants as well (Verlinden et al., ~~2018).~~2018). In the same experiment, we found that the partitioning to roots and to AMF was larger in the non-P-fertilized mesocosms as compared to the P-fertilized mesocosms (Verlinden et al., 2018). Interestingly, in the absence of AMF, plants that did not receive extra P died prematurely ~~(i.e.,~~ in pasteurized mesocosms not included in this study~~,~~ (but ~~see~~reported in Verlinden et al~~. (~~., 2018~~) and Ven et al. (2020b)). ~~).

The similar leaf P concentrations in all treatments during C2 further supports our assumption of a strong stimulation of P-acquisition through mycorrhizae in the non-P-fertilized plants. The establishment of mycorrhizal ~~(fungus-root)~~ symbioses is believed to be one of the most successful strategies to maximize the access of plant roots to available P and thus overcome P stress (Smith and Read, 2008; Sánchez-Calderón et al., 2010). The hyphal network of mycorrhizae ~~extents~~extends over a very large surface area, increasing prominently the absorbing area of roots. ~~Besides, mycorrhizal fungi improve phosphate solubility (Smith & Read 2008), and their~~Their extraradical hyphae extend beyond the P depletion zone, absorbing P that is otherwise not accessible for the plant (Plenchette et al., 2005; Roy-Bolduc and Hijri, 2011). Besides, mycorrhizal fungi improve phosphate solubility because they produce exudates that liberate P from the minerals (a.o. Smith et al., 2011; Burghelea et al., 2015; Kobae, 2019; Etesami et al., 2021; Jansa et al., 2021). For example glomalin, a glycoprotein secreted by AMF, aids the uptake of nutrients such as Fe and P that are difficult to dissolve (Miransari, 2010; Emran et al., 2017; Begum et al., 2019). Mycorrhizae thus significantly contribute to plant nutrition and to P uptake in particular (Wright et al., 2005), which in turn can positively affect leaf gas exchange rates (Smith and Read, 2008; Augé et al., 2016). ~~Here~~In our experiment, the photosynthetic parameters increased, coinciding the mycorrhization-induced improved P nutrition in the non-P-fertilized plants.

The ~~'machinery limited'~~leaf-scale responses reported here correspond well to the ecosystem-scale GPP measurements reported for the same experiment in Verlinden et al. (2018). In the first weeks, both were (very) low in the absence of P addition, but showed a sudden increase about 6 weeks after planting. Although ecosystem level GPP remained lower for the non-P fertilized treatments, the photosynthesis system ~~even~~ seemed to have fully ~~recover~~recovered, as ~~observed~~indicated by similar levels of leaf photosynthesis ~~during C2~~ among all treatments during C2. These results are in line with the study by Řezáčová et al. (2018), who reported photosynthetic upregulation following ~~C sink stimulation~~ upon the establishment of mycorrhizal symbiosis~~.~~. Also our follow-up experiment with a P gradient confirmed the important stimulating role of AMF for plant productivity and photosynthesis (see Ven et al., 2020b).

To conclude, low P availability significantly decreased photosynthetic capacity, associated with reduced concentrations of photosynthetic enzymes and pigments. In contrast to the expected increase in nutrient stress because of further depletion of the soil as the growing season progressed, nutrient stress decreased over time and for most leaf processes, pigments and enzymes under study, the fertilization effect had disappeared two months after planting. Our results point towards a key role for the AMF-symbiosis and consequent increase of P uptake in explaining the vanishing P stress.

Data availability

Data will be published on Zenodo upon acceptance.

Author contributions

S.V. designed the experiment and research; M.S.V., H.A. and A.V. conducted the field- and lab work; M.S.V. made first data analysis and draft of the manuscript; all authors provided expert advice and critically reviewed the manuscript.

Competing interests

Sara Vicca is a member of the editorial board of Biogeosciences. The peer-review process was guided by an independent editor, and the authors have also no other competing interests to declare.

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