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 an essential macronutrient for plant growth, phosphorus (P) is one of the least available nutrients in soils and 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 seasonal variation in 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 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). 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 than one third of the arable land worldwide is considered to experience 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

40   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 (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 it is unclear whether leaf traits, such as leaf nutrients,

45   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, which is needed to regenerate Ribulose 1,5-bisphosphate (RuBP) in the Calvin cycle of photosynthesis. P-deficiency therefore leads to a decrease in RuBP pool size and insufficient ATP, and consequently to a decrease in photosynthetic C assimilation. The

50   content and specific activity of 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 photosynthesis, often increases with P-stress (Zhang et al., 2014) due to low availability of P for triphosphate translocation, however decreases of leaf starch have also been observed (Halsted and Lynch, 1996).

55   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

60   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 the effect of P limitation on leaf pigments and leaf 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

65   were expected to gradually deplete the soil P.

2 Material and methods

2.1 Experimental design

A mesocosm experiment consisting of 20 (1 m × 1.2 m, 0.6 m high) insulated boxes was set up in a greenhouse

70   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

75   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

80   (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

85   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

90   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. 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

95   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 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

100   (2000) using the package 'Plantecophys' (Duursma, 2015) in R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). The 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.

105   Photosynthetic light-response curves were obtained by measurements of the net 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, the net assimilation rate at light saturation (A_{max}) and leaf dark respiration (R_{dark}, net CO_2 exchange at

110   zero light) were derived. In addition, light-induced inhibition of leaf respiration was estimated from the light response 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

115 Rubisco activity was analyzed according 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 assay of phosphoenolpyruvate carboxylase (PEPC) was coupled with the malate dehydrogenase reaction, the resulting measure of PEPC activity was expressed in µmol HCO3 m^{-2} min^{-1}.

Mono- and oligosaccharides in leaf tissue were analyzed chromatographically according to AbdElgawad et al.
120 (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 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
125 carotenoids and xanthophylls was done by a diode array detector (Shimadzu SPD-M10Avp) at four different wavelengths (420, 440, 462, 660 nm) and integrated via the software program (Shimadzu Lab Solutions Lite) 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
130 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 the sample, hyphal intersects were
135 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):

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

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 =
140 soil weight (g).

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
145 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

150 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),
155 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 the P-fertilized mesocosms (Table 1) during C1, with
mean SLA values of respectively 52.9 ± 0.9 and 33.9 ± 1.9 m² 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.

160 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 ($\pm$
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
165 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
170 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
175 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
180 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
185 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),

190   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 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

195   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).
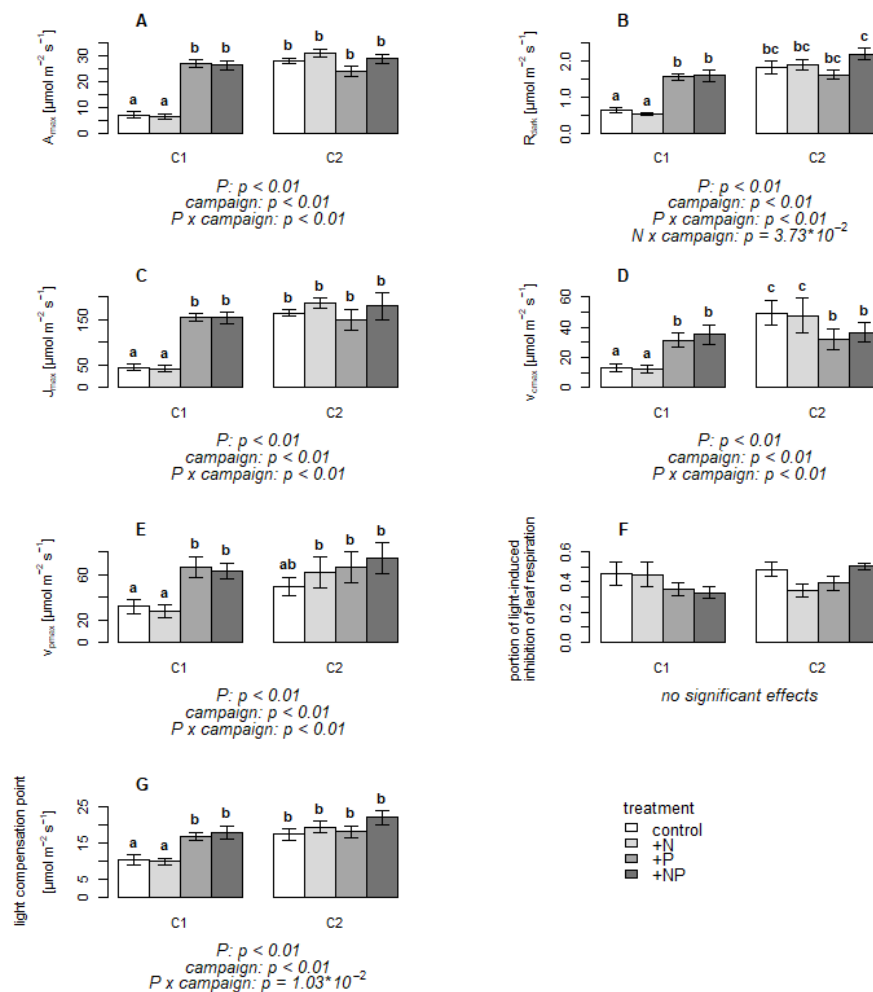
Figure 1 A-G: Means of parameters deduced from leaf CO₂ exchange measurements per treatment and campaign. Error bars
200   indicate standard error. C1: campaign 1, end of June; C2: campaign 2, end of July; control treatment: not fertilized, +N
treatment: nitrogen fertilized, +P treatment: Phosphorus fertilized, +NP treatment: both nitrogen and phosphorus fertilized.
Letters above bars indicate statistical 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 assimilation rate; J_{max} =
maximum electron transport rate; v_{cmax} = maximal rubisco carboxylation rate; v_{pmax} = maximum PEP carboxylation rate;

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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.

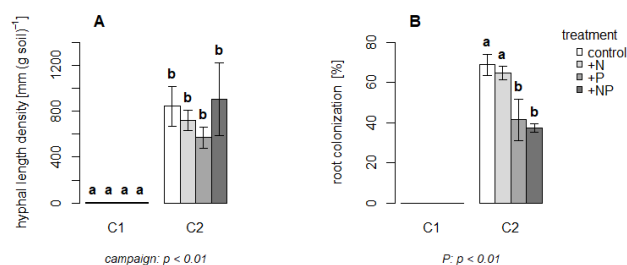
campaign	treat­ment	leaf N (g m⁻²) mean (SE)	leaf P (g m⁻²) mean (SE)	leaf N:P (-) mean (SE)	SLA mean (SE)	direct rubisco (µmol 3-PGA/m² min) mean (SE)	PEP-carboxylase (µmol HCO3/m².min) mean (SE)	total chlorophyll (mg m⁻²) mean (SE)	beta-carotene (mg m⁻²) mean (SE)	zeaxanthin (mg m⁻²) mean (SE)	violaxanthin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

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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 effects are given with p-value below the plots

4 Discussion

220 The unfertilized soil in our experiment can be assumed to be P-limited, as previously reported in Verlinden et al. (2018). Addition of P increased plant productivity, whereas N addition did not. End-of-season dry biomass reached 81 ($\pm$ 7) and 510 ($\pm$ 24) g m^{-2} for the non-P-fertilized and P-fertilized treatments, respectively (Verlinden et al., 2018). N addition ~~neither had an~~ 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

225 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 leaf N:P ratios higher than 16 (Koerselman and Meuleman, 1996) up to 20 (Güsewell, 2004) are considered to be P-limited, the high leaf N:P ratios of about 37 illustrate a clear P-limitation for the non-P-fertilized treatments in

230 C1, while ~~the~~ P-fertilized treatments ~~are~~ close to P-limitation. In C2, plants seem to have reached an optimal allocation of N and P, as shown by the optimal N:P ratio (i.e. between 9 and 18, Beauchamp and Hamilton, 1970) similar in all treatments. 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

235 (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-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 therefore generally suffer less from P limitation as compared to C$_3$ plants (Calderón-Vázquez et al.,

240 2009). This implies that, plants with absent P fertilization experienced extreme P limitation early in the season in our experiment. Nonetheless, during C2, photosynthetic parameters reached similar values among 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~~ strongly

245 increased in all treatments, both in the initially non-P-fertilized plants where chlorophyll increase was accompanied by increased photosynthesis, but also 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 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

250   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-epoxided to zeaxanthin in the xanthophyll cycle ~~in case~~ 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

255   decrease of light harvesting. In our experiment, no detectable zeaxanthin amounts were detected later in the season, indicating that stress, likely due to P-limitation, seemed 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~~ 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

260   addition had no clear effect on the leaf starch content (Table 1), but 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

265   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

270   starch from C1 to C2, possibly suggesting that plants in all treatments experienced nutrient stress during C1.

Foliar respiration rate is suppressed in the light ~~and~~ is known as the 'Kok effect', which is the abrupt decline in quantum yield of net CO_2 assimilation that occurs at very low light, often near the photosynthetic light compensation point (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

275   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

280   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~~ 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

285   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~~ of the non-P-fertilized plants, and was beneficial for productivity in the P-fertilized plants as well (Verlinden et al., 2018). Interestingly, in the absence of AMF, plants that did not receive extra P died
290 prematurely (i.e., in pasteurized mesocosms not included in this study, but see 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.,
295 2010). The hyphal network of mycorrhizae ~~extents~~ over a very large surface area, increasing prominently the absorbing area of roots. Besides, mycorrhizal fungi improve phosphate solubility (Smith & Read 2008), and 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). 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
300 rates (Smith and Read, 2008; Augé et al., 2016). Here, the photosynthetic parameters increased, coinciding the mycorrhization-induced improved P nutrition in the non-P-fertilized plants. The 'machinery-limited' photosynthesis system even seemed to fully recover as observed by similar levels of photosynthesis during C2 among all treatments. 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 .

305 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
310 in explaining the vanishing P stress.

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.

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