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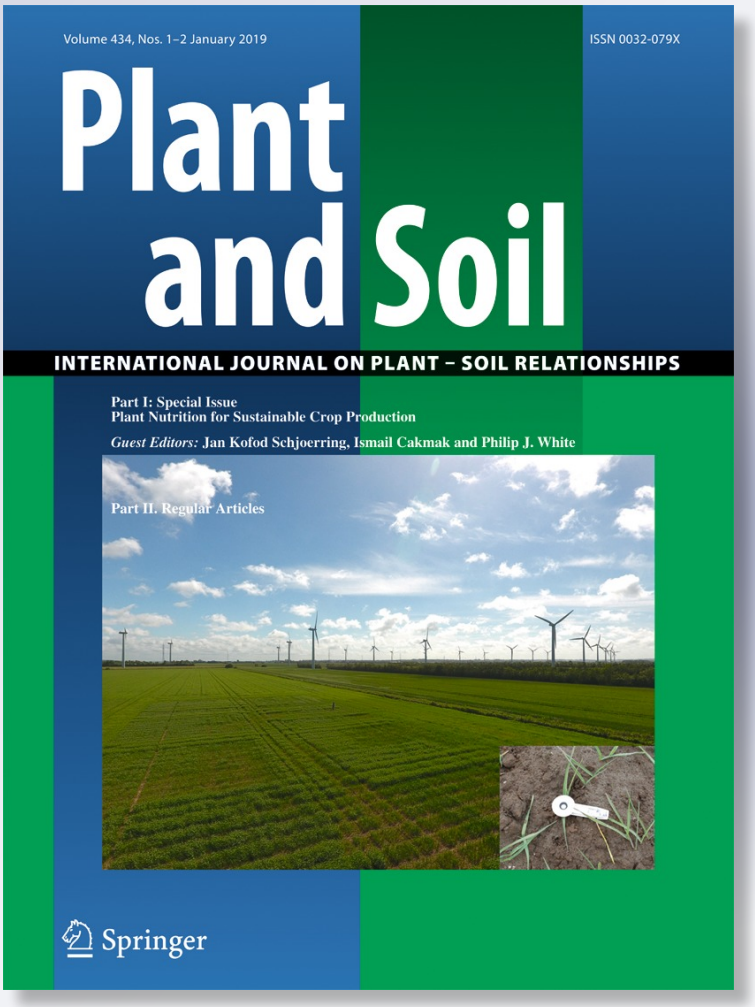

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REGULAR ARTICLE

Chlorophyll *a* fluorescence analysis can detect phosphorus deficiency under field conditions and is an effective tool to prevent grain yield reductions in spring barley (*Hordeum vulgare* L.)

Andreas Carstensen · Augusta Egelund Szameitat · Jens Frydenvang · Søren Husted

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Abstract
Background and aim Phosphorus (P) is an essential macronutrient with major impacts on global crop productivity. Recent work showed that chlorophyll *a* fluorescence analysis can be used as a sensitive indicator of latent P deficiency across different plant species. Here, we demonstrate that chlorophyll *a* fluorescence OJIP transients are a powerful tool for early detection of P deficiency directly in the field.
Methods Barley was grown in a P responsive field. One treatment received 30 kg P ha^{-1} at sowing, four treatments were fertilized with P at 26, 35, 46 or 56 days after sowing (DAS), respectively, and the final treatment did not receive any P throughout the experiment. Chlorophyll *a* fluorescence measurements, multi-elemental leaf analysis, and growth stage evaluation were performed 26, 35, 46, 56, and 69 DAS.
Results Phosphorus deficiency during early vegetative growth irreversibly affected plant development including tiller outgrowth and grain yields. However, in the present study, yield reduction could be avoided if short-term P deficiency was corrected by application of P fertilizer no later than 35 days after sowing, when plants had not yet entered the tillering stage. The chlorophyll *a* fluorescence OJIP transients were able to detect latent P deficiency in this critical phase, thereby providing an opportunity for avoiding a potential yield reduction of up to 27 hkg ha^{-1}. It was further noted, that chlorophyll *a* fluorescence analysis and P leaf tissue analysis should be performed during early vegetative growth, as probable remobilization of P within the plant during tillering and shoot differentiation masks the effects of P deficiency at the single leaf level.
Conclusions It is concluded that chlorophyll *a* fluorescence analysis provides a unique opportunity for a timely detection and correction of P deficiency under field conditions to prevent yield reductions.

Keywords Phosphorus deficiency · Chlorophyll *a* fluorescence · Tillering · Field experiment · Barley

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Introduction

Phosphorus (P) is an essential plant nutrient and is needed by all plants in order to complete a full life cycle. It is a key element in generation of ATP and NADPH, synthesis of nucleic acids, synthesis and stability of membranes, enzyme activation/inactivation, glycolysis, respiration and photosynthesis (Hawkesford et al. 2011). During photosynthesis, carbon is fixed and exported from the

chloroplasts into the cytosol as triose phosphate, where it is converted to sucrose releasing orthophosphate (Pi). During P deficiency, this export of triose-phosphates is decreased, retaining more photosynthates in the chloroplast stroma, where it is converted to starch. As a consequence, low Pi-levels switch the carbon flow to starch accumulation which reduces CO_2 assimilation and biomass productivity (Rychter and Rao 2005).

The symptoms of P deficiency includes reduction in leaf expansion and number of leaves, suppression of tillering, decreased shoot/root ratio, early senescence, and a reduction in the size and number of flowers and grains (Hammond and White 2008). Prolonged P deficiency may further result in accumulation of anthocyanins and other secondary metabolites, which recycles substantial amounts of Pi from phosphorylated precursors, thereby assisting P deficient plants to cope with environmental challenges and to prevent photoinhibition (Ticconi and Abel 2004). Adequate P supply is especially important early in the season, as plants might not recover from being temporarily P deficient during seedling development. Low P availability later in the season usually has a much smaller impact on plant development (Grant et al. 2001).

One of the main structural changes in response to low P availability is the process of tiller development. Tillering is a key component of yield for major cereals, where the number of tillers can be adjusted either by reducing the outgrowth of new tillers or increase tiller senescence. The processes are highly dynamic and are regulated by a complex network of genetic, environmental and hormonal factors (del Moral and del Moral 1995). A complex system of hormonal interactions control tiller formation during P deficiency where auxin and strigolactone (tillering suppressors), and cytokinin (tillering promotor) play key roles (Kebrom et al. 2013).

It has been estimated that 30% of the world's agricultural soils are P deficient and require P fertilization to secure crop productivity and quality (MacDonald et al. 2011). However, rock phosphate, which is the main source of P fertilizers, is a finite natural resource, which needs to be utilized efficiently in agriculture (Baker et al. 2015; Cordell et al. 2009). In addition, only a few countries control the vast majority of the known reserves, which potentially turns P resource management into a political and strategic issue (Edixhoven et al. 2013; Gilbert 2009). As fertilizer P is rapidly immobilised in soil, excessive P application is often required to ensure ample P availability to plants. As a consequence, less than 20% of the added P fertilizer is typically taken up by the crop (Cordell and White 2015). Currently, some parts of the world overuse P fertilization, contributing to the eutrophication of lakes and seas, while P depletion results in severe yield limitations elsewhere (Baker et al. 2015; MacDonald et al. 2011).

Different analytical techniques are being used in order to test the P status of agricultural soils, which includes a wide range of soil extraction procedures to reflect the potentially plant available fraction of P. However, as P is highly immobile in soils and also influenced by a range of parameters not accounted for in a soil analysis, the correlation between extracted P and plant uptake is often poor, especially if compared across a range of different soil types (Mundus et al. 2017). It is attractive to change the approach from estimating what might be plant available in the soil P pool, to measure the plant P status directly, as this would reveal information about true plant P uptake and indicate if acute P supplementation is required. Analysing changes in biochemical performance during P deficiency might be a better approach for estimating the P status of plants, long before any visual symptoms appear.

Measuring chlorophyll *a* fluorescence is a rapid and non-invasive probe of plant photo-chemistry, and the technique has been used for decades to monitor a wide range of biotic and abiotic stresses of algae and higher plants (Kalaji et al. 2017; Guo and Tan 2015; Brestic and Zivcak 2013). Recently, it has been shown that the shape of the so-called I-step of the chlorophyll *a* fluorescence transient (OJIP transient) can be used to determine latent P deficiency across different plant species (Frydenvang et al. 2015). The correlation between the appearance of the I-step and the bioactive P pool has been presented recently (Carstensen et al. 2018), and it has been shown that P deficiency limits the pool of Pi in the chloroplasts, which is a substrate for ATP formation. As a consequence, the rate of ATP synthase will decrease and protons will accumulate in the thylakoid lumen causing acidification, which finally will decrease the oxidation of the plastoquinol (PQH_2) pool at the cytochrome *b6f* (Cyt *b6f*) complex in the electron transport chain. The decreased oxidation will change the flow of electrons towards photosystem I (PSI), which is reflected by the shape of the I-step.

The objective of this paper was to evaluate the use of OJIP transients directly in the field to estimate the P status of barley, and measure the effects of P fertilization supplied to plants at different stages during early

vegetative growth. We wanted to test if the method could be used for a timely diagnosis of short-term P deficiency and prevent irreversible damage to seedling growth, tiller development and grain yields.

Materials and methods

Field experiment

A location in Northern Jutland, Denmark (N: 57°7'54.706; E: 9°33'24.629), was selected for a field trial in the summer of 2017, as the location previously has been shown to respond to P fertilization based on experience from the national agriculture advisory service (www.seges.dk). Before sowing and fertilization, subsamples of soil from the top 25 cm were taken, mixed, air dried, and sieved to below 2 mm. Soil texture, SOM, pH, Olsen-P, DGT-P, ammonium acetate extractable Mg, and K, were analysed using Danish standard soil testing procedures (Sørensen and Bülow-Olsen 1994), at a commercial soil testing laboratory (Agrolab; Table 1). Six plots of 67.5 m^2 (4.5*15 m) were placed in a complete randomized block design with four replicates (24 plots total). The entire experiment received N-K (19–0-15) fertilization with S, Mg, and B in amounts corresponding to 110 kg N ha^{-1}. At sowing, four plots (treatment P + 0DAS) received triple super phosphate (0–20-0) corresponding to 30 kg P ha^{-1} placed 4 cm below the seeds. The crop was spring barley (*Hordeum vulgare* L. cv. RGT Planet) sown with 300 seeds m^{-2} in 3 cm depth. Four treatments received P 26, 35, 46 or 56 days after sowing (DAS), respectively, by broad spreading triple super phosphate manually in the same amount as described above. The final treatment did not receive any P during the whole experiment. Chlorophyll *a* fluorescence measurements, leaf sampling and growth stage evaluation (according to Zadoks code; Zadoks et al. 1974) were performed 26, 35, 46, 56, and 69 DAS. At full maturity, 12.5 m^2 from each plot were harvested to determine grain yield.

Table 1 Soil properties at the experimental field site. SOM: Soil organic matter. The results are means ±SEM ($n = 4$)

Soil content	Value
SOM (%)	2.1
Clay (%)	6.3
Silt (%)	3.9
Fine sand (%)	72.1
Coarse sand (%)	15.6
pH ($CaCl_2$)	4.7 ± 0.1
Olsen-P (mg P/kg soil)	23.0 ± 2.0
C_{DGT} (μg P/L)	62.8 ± 7.0
Potassium (mg K/kg soil)	54.2 ± 1.1
Magnesium (mg Mg/kg soil)	45.4 ± 1.0

Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence transients were obtained from the youngest fully expanded leaves (YFELs) using a Handy PEA chlorophyll fluorometer (Hansatech Instruments, UK). Six measurements were performed on each plot and were averaged. The YFEL mid-section was dark-adapted for at least 25 min before measurements using Hansatech leaf clips. A short non-actinic light flash was applied to the leaf to adjust the detector gain just prior to measuring the fluorescence transients, and each leaf sample was illuminated with continuous saturating actinic light (3000 μmol photons m^{-2} s^{-1}) from three LEDs. The fluorescence transients were recorded using a PIN photodiode for a period of 10 s. All graphical illustrations of the transients were double-normalized between F_0 and F_M to give the relative variable fluorescence at time t as follows: $V(t) = [\text{Fluorescence}(t)\text{-}F_0]/(F_M\text{-}F_0)$.

Generating the phosphorus prediction model

To generate a P status prediction model for the field measurements, we used an approach similar to that presented in Frydenvang et al. (2015). However, we implemented a few additional pre-processing steps, and a Partial Least Squares Discriminant Analysis (PLSDA) model (Barker and Rayens 2003; Brereton and Lloyd 2014) was used to differentiate between healthy and P deficient plants. The pre-processing of all OJIP transients was performed in Python 3.6.3 (Python Software Foundation - available at http://www.python.org, acquired using the Anaconda Distribution - available at http://www.anaconda.com), using the packages SciPy (Jones et al. 2001) and Pandas (McKinney 2010). The PLSDA model was made using Matlab R2017b (Mathworks) and PLS_Toolbox 8.6 (Eigenvector Research Inc.)

For the prediction of plant P status, all OJIP transients were smoothed using a spline fit (from the SciPy

'Interpolate' package) and resampled to obtain equidistant data points based on the logarithm of the time, and then normalized by F_0 and reset to origo. Based on a selected reference OJIP transient from a healthy barley plant in the calibration dataset, all transients were first-order-corrected to this reference curve to minimize lower order variations between measurements (Frydenvang et al. 2015). The temporal location of the J- and I-P sections, respectively, of individual OJIP transients were then likewise matched to the reference curve. Finally, the transients were differentiated, based on a spline-fit of the resampled transient, to enhance the non-linear features of the OJIP transients.

To generate the PLSDA model, a calibration dataset consisting of OJIP transients from plants cultivated at different P concentrations in a greenhouse previously presented in Frydenvang et al. (2015) were collected. This yielded a calibration dataset of 618 OJIP transients, all with a known P concentration determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Outliers were detected and removed based on interpretations of plots showing PLSDA principal components as well as model residual vs. hotelling T^2 (Hotelling 1931) parameters. In total, 91 transients were removed as outliers, leaving a calibration dataset of 527 OJIP transients. A 3-fold cross-validation was used to optimize the number of components in the PLSDA model, optimized to differentiate between transients from plants with a P concentration below 2500 μg P g^{-1} DW (low P class), and above 3000 μg P g^{-1} DW (healthy P class). From this, 4 PLSDA components were estimated to produce the most predictive model. This model, built on measurements from plants grown in pots and hydroponic units in a green house, was used to predict the P status of the field-measured OJIP-transients presented in this study. The field measured OJIP transients were not included in the modelling effort, but was subjected to identical data pre-processing as the calibration data. The reported P prediction number represents the likelihood of a plant to fall into the "healthy" group in the PLSDA analysis.

Elemental analyses

After chlorophyll *a* fluorescence measurements, the YFELs were collected and oven-dried at 50 °C until no differences in weight was observed (complete dryness) and digested with ultra-pure acids (70% HNO_3 and 30% H_2O_2) at 240 °C and 200 bars for 15 min in a pressurized microwave oven (Ultrawave, Milestone Inc., Italy). Leaf element concentrations were determined using ICP-OES (5100, Agilent Technologies, USA) equipped with a Meinhard nebulizer and a cyclonic spray chamber, following the protocol from Hansen et al. (2009). Certified reference material [NIST1515, apple (*Malus* ssp.) leaf, National Institute of Standards and Technology, USA] was included to evaluate data quality. Data were processed using Agilent ICP Expert software.

Data analyses

Statistical analysis was performed using R (version 3.4.1). Each mean was compared using one-way ANOVA analysis with one random effect and Tukey's multiple pairwise comparison test. Samples were considered significantly different at $P < 0.05$. The graphical illustrations were made in GraphPad Prism 6.0 for Windows.

Results

Field experiment

A field in Northern Jutland, Denmark, was chosen as the experimental site, which previously has been shown to respond to P fertilization. Before crop establishment, the basic soil properties were determined, classifying the field as a sandy soil with Olsen-P and DGT-*P* values of 23.0 ± 2.0 and 62.8 ± 7.0, respectively (Table 1). To test the effect of short-term P deficiency on plant development and yields, six different treatments were conducted: Treatment 1 (P + 0DAS) received P at sowing, treatment 2–5 received P 26, 35, 46 or 56 days after sowing (DAS), respectively, and finally treatment 6 (P-) which did not receive any P throughout the whole growth period (Table 2). Chlorophyll *a* fluorescence transients, elemental composition, and growth stage development were recorded at 26, 35, 46, 56, and 69 DAS.

Plant development

There were no visual differences between the treatments at 26DAS, but only nine days later (35DAS), the P + 0DAS treatment could be visually distinguished from the remaining five treatments by an increased biomass

Table 2 The six different treatments used in the field trial ($n = 4$). 30 kg P ha^{-1} was placed at sowing for treatment 1, whereas treatment 2–5 received 30 kg P ha^{-1} (broad spread) at 26, 35, 46 or 56 days after sowing (DAS), respectively. Treatment 6 did not receive any P throughout the whole growth period

Treatment	P fertilization	DAS	Date
1. P + 0DAS	30 kg P ha^{-1} placed at sowing	0	6 April
2. P + 26DAS	30 kg P ha^{-1} broad spread	26	2 May
3. P + 35DAS	30 kg P ha^{-1} broad spread	35	11 May
4. P + 46DAS	30 kg P ha^{-1} broad spread	46	22 May
5. P + 56DAS	30 kg P ha^{-1} broad spread	56	1 June
6. P-	No P added	–	–

production (data not shown). At 46DAS, also P + 26DAS and P + 35DAS could be separated from the unfertilized plots (P- and P + 56DAS), but without a clear distinction between treatment P + 26DAS and P + 35DAS (Supplementary Fig. S1). The visual differences between the treatments were even more pronounced at 56 DAS (Fig. 1). Treatment P + 0DAS, P + 26DAS, P + 35DAS, and P + 46DAS could visually be separated, whereas there were no visual difference between P + 56DAS (which received P the same day as the photo was taken) and P-.

The visual appearances were confirmed by the growth stage development using Zadoks scale (Table 3; Zadoks et al. 1974). At 26 DAS, one leaf was developed for all six treatments (Zadoks stage 11), and nine days later (35 DAS), P + 0DAS had developed three leaves (Zadoks stage 13) whereas the remaining treatments had developed two leaves (Zadoks stage 12). At 46 DAS, the three treatments which received P already (P + 0DAS, P + 26DAS, and P + 35DAS) had entered the tillering stage (Zadoks stage 20–21), whereas the remaining treatments were still in the seedling development stage (Zadoks stage 13). At 56 DAS the four treatments receiving P (P + 0DAS, P + 26DAS, P + 35DAS, and P + 46DAS) had now entered the stem elongation stage (Zadoks stage 31–32), including treatment P + 46DAS which only received P ten days earlier. The two treatments not receiving any P yet (P + 56DAS and P-) were still in the seedling development stage (Zadoks stage 14–15). At the final day of measurement (69DAS) all the treatments (including P- which did not receive any P) were at the booting stage (Zadoks stage 45–49), despite the fact that P + 0DAS and P- significantly differed in plant height (51.0 ± 0.5 and 30.5 ± 0.7 cm, respectively) and tiller outgrowth (7.0 ± 1.5 and 1.3 ± 0.3, respectively; Table 3).

Phosphorus concentration in leaf tissue

To further confirm plant responses to the added P fertilization, the total P concentration in YFEL was determined at 26, 35, 46, 56, and 69 DAS (Fig. 2). There were no significant differences between any of the treatments at 26 DAS (where only treatment P + 0DAS had received P), and all six treatments showed values above the critical threshold level at 2000 μg P g^{-1} DW (Reuter and Robinson 1997). Nine days later (35 DAS) marked differences in leaf P content were observed. The P +

Fig. 1 Photos of the six different treatments 56 days after sowing (DAS). Treatment P + 46DAS, P + 35DAS, P + 26DAS and P + 0DAS could be visual separated by increasing biomass, whereas there were no visual differences between P + 56DAS and P-. The photographs were taken prior sampling and P addition to treatment P + 56DAS

Table 3 Growth stage development for the six treatments at 26, 35, 46, 56 and 69 days after sowing (DAS), including total plant height and number of tillers at 69 DAS. Growth stage evaluation (using Zadoks scale) was determined visually, whereas plant height and number of tillers are means ± SEM ($n = 4$), and different letters represent statistically significant changes ($P < 0.05$) using a one-way ANOVA analysis and Tukey's multiple comparison test

Treatment	Growth stage					Height (cm)	Tillers
	26 DAS	35 DAS	46 DAS	56 DAS	69 DAS	69 DAS	69 DAS
1. P + 0DAS	11	13	21	32	49	$51.0^{a} \pm 0.5$	$7.0^{a} \pm 1.5$
2. P + 26DAS	11	12	20	32	49	$50.2^{a} \pm 0.8$	$5.3^{a} \pm 0.5$
3. P + 35DAS	11	12	20	32	49	$40.0^{b} \pm 0.8$	$3.8^{b} \pm 0.3$
4. P + 46DAS	11	12	13	31	47	$30.1^{c} \pm 0.5$	$3.0^{b} \pm 0.4$
5. P + 56DAS	11	12	13	15	47	$29.8^{c} \pm 0.8$	$1.8^{c} \pm 0.3$
6. P-	11	12	13	14	45	$30.5^{c} \pm 0.7$	$1.3^{c} \pm 0.3$

0DAS treatment was still above the critical threshold (close to 3500 μg P g^{-1} DW), whereas the remaining five treatments were significant lower. Interestingly, the treatment receiving P 9 days earlier (P + 26DAS) indicated a P response with values just around the threshold at 2000 μg P g^{-1} DW, whereas all the non P fertilized treatments were significantly lower. Eleven days later (46DAS), P + 26DAS (which received P 20 days earlier) was above the P threshold and had similar P levels compared to the control treatment (P + 0DAS) at 3000 μg P g^{-1} DW. The treatment receiving P fertilizer 11 days earlier (P + 35DAS) clearly showed a fertilizer response with values just below 2000 μg P g^{-1} DW, whereas the three treatments not yet fertilized with P (P + 46DAS, P + 56DAS, and P-) had significant lower values around 1000 μg P g^{-1} DW, which is considered as severely P deficient (Reuter and Robinson 1997). After yet another 10 days (56DAS) the significant differences between the treatments were no longer present. All of the six treatments showed P concentrations above the critical threshold at 2000 μg P g^{-1} DW, including the two treatments which not yet had received any P fertilization (P + 56DAS and P-). The same tendency was present at the final measuring day (69DAS). Here, the treatment receiving P 13 days earlier (P + 56DAS) showed the largest P content around 2500 μg P g^{-1} DW, whereas the treatment without any P added (P-) showed values just below the critical threshold at 2000 μg P g^{-1} DW (Fig. 2).

Diagnosing phosphorus deficiency using chlorophyll *a* fluorescence transients

To test if P deficiency could be diagnosed using chlorophyll *a* fluorescence under field conditions, the OJIP transients were measured 26, 35, 46, 56, and 69 DAS (Fig. 3; Supplementary Fig. S2). Using the P prediction model first described in Frydenvang et al. (2015) (see materials and methods for further details), we calculated P prediction values from the OJIP transients. Values below 0.4 were classified as P deficient corresponding to the 2000 μg P g^{-1} DW deficiency threshold limit (marked with "*" in Fig. 2). The P prediction model essentially describes the shape of the I-step, as a straightening of this step previously has been shown to specifically reveal P deficiency of barley plants (Carstensen et al. 2018; Frydenvang et al. 2015)

There were no visual differences in the shape of the I-step between the treatments at 26 DAS, which was confirmed by the corresponding P prediction values >0.5 (Supplementary Fig. S2b). This was in agreement with the measured leaf P concentration at 3000 μg P g^{-1} DW for all six treatments (Fig. 2). After 35 DAS, P + 0DAS still showed a distinct I-step with a corresponding P prediction value at 0.70 (indicating no P deficiency), whereas treatment P + 26DAS and P- revealed a marked straightening of the I-step, with corresponding P prediction values at 0.21 and 0.07, respectively, indicating P deficiency (Supplementary Fig. S2d). This classification was again in agreement with the measured P concentration for P + 26DAS and P- (2000 and 1600 μg P g^{-1} DW, respectively), and both methods reflect the P fertilizer response for treatment P + 26DAS, which received P nine days prior to the measurement. At 46 DAS, the four different treatments could be clearly separated by the shape of the I-step of the OJIP transients (Fig. 3b). The number of days with available P directly corresponds to the observed tendency going from a pronounced I-step for treatment P + 0DAS to an almost complete suppression of the I-step for treatment P-. The P prediction values similarly correspond to the tissue P concentration, diagnosing treatment P + 0DAS and P +

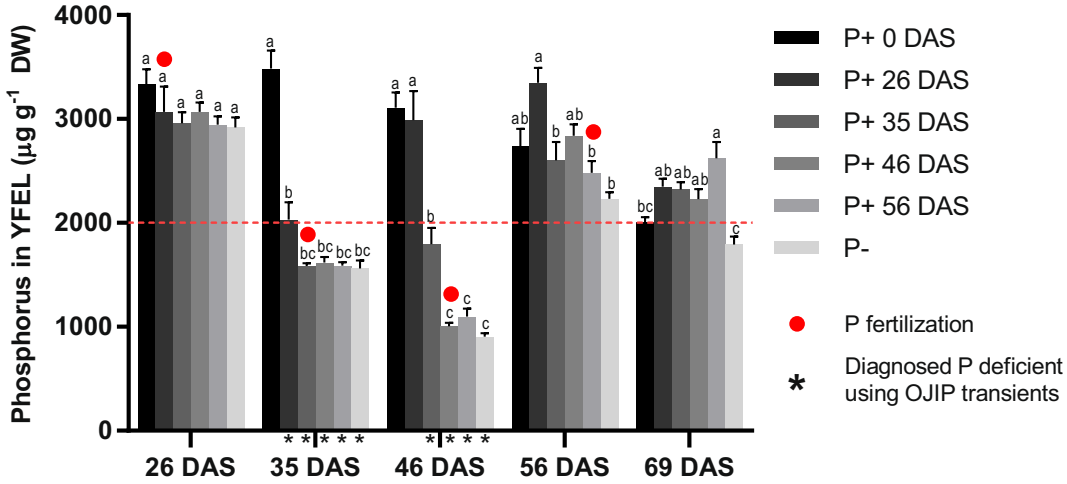

Fig. 2 Development in leaf phosphorus concentrations in barley when phosphorus was added at different time points. Phosphorus was placed at sowing (P + 0DAS) or broad spread at 26, 35, 46 or 56 days after sowing (DAS), respectively. The P- treatment did not receive any phosphorus throughout the growth period. The total phosphorus concentration in the youngest fully expanded leaf (YFEL) was determined at 26, 35, 46, 56 and 69 DAS, respectively. The red dotted line indicates the critical threshold concentration for phosphorus at 2000 µg P g^{-1} DW, the red dots indicate phosphorus fertilization, and "*" indicates phosphorus deficient plants deduced from chlorophyll *a* fluorescence transients. The results are means ± SEM ($n = 4$), and different letters represent statistically significant changes ($P < 0.05$) using a one-way ANOVA analysis and Tukey's multiple comparison test within each time point

26DAS as P sufficient with P prediction values at 0.85 and 0.59, respectively, treatment P + 35DAS with a P prediction value at 0.38, and finally P- with a P prediction value at −0.04 (Fig. 2; Fig. 3). The sudden disappearance of P deficiency at 56 and 69 DAS for all treatments, as reflected by the total P concentrations, were also confirmed by the OJIP transients. The prediction model could differentiate between the P treatments, but none of them were classified as P deficient (Supplementary Fig. S2f;h). There were no significant differences between the treatments when using the many standard parameters from the OJIP transient, including F_0, F_M, F_V/F_M and performance index (data no shown; Strasser et al. 2000).

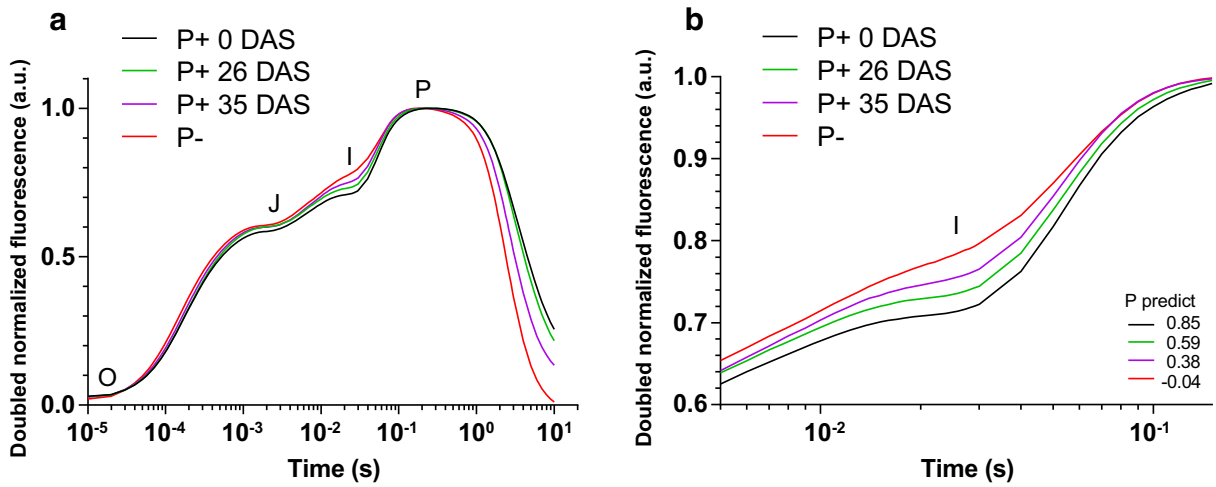

Fig. 3 OJIP transients recorded from the youngest fully expanded leaves of the four different treatments 46 days after sowing (DAS). **a**, the complete O-J-I-P fluorescence transients. **b**, zoomed in at the so-called I-step of the OJIP transients, where the four different treatments were clearly separated. The transients were measured for 10 s, averaged ($n = 4$, each with >4 technical replicates), and doubled-normalized between F_0 and F_M

The apparent relationship between the P prediction values originating from the OJIP transients and the P concentration in the YFELs was confirmed by correlating the values, revealing a clear correlation between the two methods with an R^2 value at 0.61 (Fig. 4). When using the P threshold at 2000 μg g^{-1} DW, 108 out of 120 data points were correctly classified (90%).

Effect of short-term phosphorus deficiency on grain yield

After full maturity (Zadoks stage 92), 12.5 m^2 of each plot were harvested to determine grain yields (Fig. 5). There were no significant differences between the treatment receiving P at sowing (P + 0DAS), and the treatments receiving P at 26 or 35 DAS (P + 26DAS and P + 35DAS, respectively). The remaining three treatments all showed significantly lower yields. However, the treatments receiving P at 46 or 56 DAS (P + 46DAS and P + 56DAS, respectively) did respond to the P fertilization, as the yields were significantly larger than for the P- treatment. The total difference in grain yield between the treatment receiving P at sowing (P + 0DAS) and the treatment not receiving P fertilizer (P-) was 27 hkg ha^{-1}.

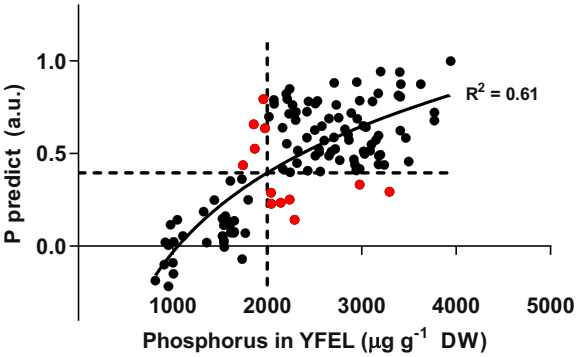

Fig. 4 Correlation between measured phosphorus concentration in the youngest fully developed leaves (YFEL) and the P prediction values estimated from the OJIP transients using a multivariate model based on entirely independent calibration data. The dataset consists of measurements from 24 plots (six treatments with four replicates) measured five times (26, 35, 46, 56, and 69 days after sowing) giving a total of 120 data points. The two dotted lines indicate the P threshold at 2000 μg g^{-1} DW, and the twelve red dots represent data points that were classified wrong according to this threshold

Discussion

Plant response to phosphorus application at different time points

Plants grown at the experimental site showed a clear response to P fertilization, although soil analyses by Olsen-P and DGT-P failed to classify the soils as potentially P responsive, using the critical threshold levels of 20 mg P/kg soil (Knudsen and Østergaard 2012) and 65 μg P L^{-1} (Mason et al. 2010; Mundus et al. 2017), respectively. Already at 35 DAS (Zadoks stage 13), the treatment receiving P at sowing (P + 0DAS) could be visually separated from the other treatments based on an increased biomass production (data not shown). The P response of treatments P + 26DAS and P + 35DAS were also visually confirmed at 46DAS (Supplementary Fig. S1), and at 56 DAS, where plots with the treatments P + 0DAS, P + 26DAS, P + 35DAS, and P + 46DAS could be separated by visual inspection, and they grouped distinctively from the two remaining treatments which had not yet received P (P + 56DAS and P-; Fig. 1). The response to P deficiency was further evident by the total grain yield, where a significant difference of 27 hkg ha^{-1} was obtained between the treatment fertilized with P at sowing (P + 0DAS) and the non P fertilized treatment (P-; Fig. 5).

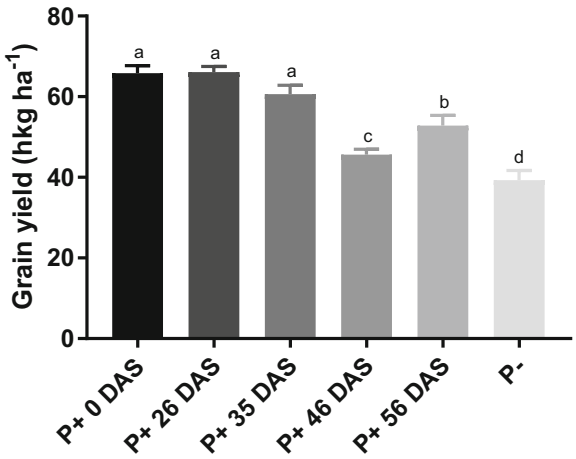

Fig. 5 Grain yield of the six treatments after harvest. After full maturity (Zadoks stage 92), the barley plants were harvested to determine the final grain yield. The results are means ± SEM (n = 4), and the letters represent statistically significant changes (P < 0.05) using a one-way ANOVA analysis and Tukey's multiple comparison test

The four treatments receiving P by broad spreading triple super phosphate on top of the soil after crop establishment (P + 26DAS, P + 35DAS, P + 46DAS, and P + 56DAS) all showed a clear response to P fertilization both considering the growth stage development (Table 3), total P concentrations in YFEL (Fig. 2), OJIP transients (Fig. 3; Supplementary Fig. S2), and total grain yields (Fig. 5). Phosphate from the fertilizer granules obviously moved into the top soil where it was dissolved and subsequently assimilated by the plant roots. Nine days after P addition to treatment P + 26DAS, a significant P response was reflected in the total leaf tissue P concentrations (Fig. 2; 35DAS) and 20 days after P addition, the P concentration was back at the control level (Fig. 2; 46DAS). The same was evident for treatment P + 35DAS, showing a significant fertilizer response eleven days after P application (Fig. 2; 46DAS). All five P treated treatments (P + 0DAS, P + 26DAS, P + 35DAS, P + 46DAS, and P + 56DAS) responded to the P fertilization with significant larger grain yields compared to the P- treatment (Fig. 5).

Chlorophyll *a* fluorescence transients are able to detect phosphorus deficiency under field conditions

Chlorophyll *a* fluorescence transients (OJIP transients) were measured prior to P fertilization for all treatments during the period from 26 to 69 DAS (Fig. 3, Supplementary Fig. S2). Studies have previously shown that the curvature of the I-step of OJIP transient can be used as a proxy to determine the bioactive P concentration in plants (Frydenvang et al. 2015) and the correlation between the appearance of the I-step and the bioactive P pool in leaves has been presented recently (Carstensen et al. 2018). Clear differences at the I-step were observed between the treatments (Fig. 3, Supplementary Fig. S2), but to avoid a pure visual classification of changes in the I-step curvature, we used a mathematical approach similar to that presented in Frydenvang et al. (2015) to calculate a P predict value for each transient (see Material and Methods for further details).

When correlating the P predict values (120 data points total) with the corresponding P concentration of the measured leaf determined by ICP-OES, a clear correlation was obtained with a R^2 value at 0.61 (Fig. 4). In particular, it was evident that a P predict value below 0.40 corresponded to the critical P deficient YFEL level at 2000 μg g^{-1} DW (Reuter and Robinson 1997). Using this threshold, five measurements were classified as false positives, seven measurements were classified as false negatives, and the remaining 108 measurements were classified correctly (90%). Interestingly, the measurements were obtained across age and growth stages, where the first measurements were taken at 26 DAS (Zadoks stage 11) and the latest measurements at 69 DAS (Zadoks stage 49), which does not seem to influence the P classification (Fig. 2; Fig. 3; Supplementary Fig. S2). Also, it is important to highlight that the reported P prediction values originate from applying a model that was calibrated on an entirely independent calibration dataset, consisting of measurements from different plant species grown in a greenhouse. The observed correlation between the P predict values and the P content in the YFEL underlines the validity and robustness of the model, a prediction that is likely to be further improved by future inclusion of measurements from field-grown plants to the calibration dataset.

The OJIP transients and the corresponding P predict values correctly classified the five P deficient treatments at 35 DAS (Fig. 2). As P fertilization within 35 DAS was still in time to avoid a yield loss (Fig. 5), OJIP transients showed to be a strong method for diagnosing short-term P deficiency directly in the field. Especially the fast analysis seems to be a strong advantage of this method. After 25 min of dark adaptation, it only takes a few seconds to collect the full OJIP transient. The P prediction model can process the data immediately, providing instantaneously P predict values of the measured plant. This is a major advantage relative to the classical plant tissue analysis, which includes sampling, drying, digesting, analysing and data processing to obtain the test result. If the analysis is handled by a commercial laboratory, the total time consumption is frequently 2–3 weeks, which this study clearly shows is way too long if yield losses are to be avoided. Growth stage development was also able to reveal the different P treatments (Table 3), however, as a wide range of climatic factors and nutrient limitations can affect plant growth, it is considered as a rather unspecific proxy for P deficiency. In addition, chlorophyll *a* fluorescence allows an earlier detection of latent P deficiency, which is fundamental in remediation of P deficiency to restore plant growth and development.

It was further noted that the decrease in fluorescence intensity after reaching maximum fluorescence (P-step at approximately 200 ms to 10 s; Fig. 3a) was also able to

differentiate between the four treatments. A faster decrease in fluorescence emission from maximum fluorescence has been correlated with increased thylakoid lumen acidification, and could likewise be a valuable proxy of P deficiency (Carstensen et al. 2018; Goltsev et al. 2016).

High phosphorus availability is crucial for seedling development and tillering

Tillering is a key determinant of harvest yields for cereals, and suppression of tillering is a well-known symptom of P deficiency (Elliott et al. 1997; Hammond and White 2008; Hoppo et al. 1999). In the present study, seedling growth and tiller outgrowth differed significantly between the six treatments (Table 3). Despite similar growth stages at the final measuring day, total plant height and tiller outgrowth was significantly different between P + 0DAS and P- (51.0 ± 0.5 and 30.5 ± 0.7 cm in total plant height, and 7.0 ± 1.5 and 1.3 ± 0.3 in tiller formation, respectively; Table 3). After 56 DAS, the two non P fertilized treatments (P + 56DAS and P-) were still in the seedling development stage, whereas the four P fertilized treatments (P + 0DAS, P + 26DAS, P + 35DAS, and P + 46DAS) had already passed the tillering stage and were now in the stem elongation stage. During the following 13 days (to 69DAS), P- went directly from the seedling stage (Zadoks stage 14) to the booting stage (Zadoks stage 45), and were suddenly at the same growth stage as the remaining 5 treatments (Table 3). Phosphorus deficiency during seedling growth essentially prevented the induction of tillering.

A developing bud is supplied with photoassimilates from its parent tiller until it has established sufficient leaf area to become an independent source for carbohydrates (Kirby and Jones 1977). A balance between number and vigour of tillers is therefore essential to avoid a decrease in yield, and consequently it is crucial to secure a sufficient amount of plant available P early in the seedling development stage, to allow barley plants to enter the tillering stage as soon as possible. Typically, tiller numbers increase during the tillering stage (Zadoks stage 20–30), reaching a peak during the elongation stage (Zadoks stage 30–40), and finally declining to a stable level during the ear emergence stage (Zadoks stage 50–60; Kirby et al. 1985; Simmons et al. 1982). The competition for photoassimilates and nutrients between the tillers is believed to be the main reason for tiller dieback, rather than an increased competition for light (Lauer and Simmons 1988). Due to low P supply, plants in the P- treatment skipped the tillering stage and entered the booting stage directly to increase the chances for completing a full life cycle for the single tiller produced, rather than generating additional tillers with the risk of subsequent die-back later in the season due to an insufficient P supply.

As early as the 1920s the importance of early season P nutrition was highlighted. Brenchley (1929) observed that the P supply for barley was most critical between 14 and 28 days after crop establishment as the tillering stage was initiated after 4 weeks of growth. Also Green et al. (1973) observed that P deficiency in the first 24 days caused reduced plant growth and tillering of barley, and resupply with P after 24 days did not result in increased biomass production. Decreased tillering in wheat as a response to low P supply was further observed by Rodríguez et al. (1998, 1999), concluding that P directly alters number of tillers by slowing the emergence of leaves on the main stem and by reducing the maximum rate of tiller emergence, which could be interpreted as a response to an increased competition for P within the plant (Rodríguez et al. 1999).

In barley, bud outgrowth is induced sequentially after the three leaf stage (Zadoks stage 13) and is highly dynamic (Alqudah et al. 2016). Inorganic P availability is known to alter the hormone balance, sensitivity, and transport, and the change in hormonal balance is believed to be the main cause of decreased tillering during P deficiency (Alqudah et al. 2016; Evers and Vos 2013; Hussien et al. 2014; Kebrom et al. 2013).

Despite the gradual decrease in tiller formation when applying P at 0, 26 or 35 DAS, (7.0 ± 1.5, 5.3 ± 0.5, and 3.8 ± 0.3 tillers, respectively; Table 3) there were no significant differences in total grain yield between the treatments that received P within 35 DAS (P + 0DAS, P + 26DAS, and P + 35DAS; Fig. 5). However, waiting to apply P to 46 or 56 DAS caused a significant reduction in grain yield (with 3.0 ± 0.4 and 1.8 ± 0.3 tillers, respectively). P + 46DAS or P + 56DAS still managed to respond to the added P fertilizer, as the grain yield was significant larger than P-, but the P application was too late to compensate for the damaging effects of short-term P deficiency during seedling development. It is noteworthy that the ability to adjust tillering according to the plant availability of P appears to be an irreversible process, where tillering is gradually decreased relative to the duration of P deficiency. Tillers that emerge late in season will not produce any grain and acts as a sink for nutrients and photoassimilates (Hussien et al. 2014). Decreased tillering is generally considered more critical for the grain yield in

barley as each spikelet only contains one floret, whereas wheat can partly compensate for low tiller numbers by increasing the number of florets per spikelet and thereby also the number of grains per ear (Arisnabarreta and Miralles 2008). As a consequence, it might be possible to correct P deficiency at a later stage in wheat and other cereals, however, this remain to be investigated.

Phosphorus deficiency spontaneously disappears in the reproductive stage

No differences in total P concentration were observed between the treatments at 26DAS, all showing sufficient *P* values around 3000 μg P g^{-1} DW (Fig. 2). This indicates that the primary P supply at this stage originated from the seed, which can sustain maximal growth of cereal seedlings for several weeks after germination (White and Veneklaas 2012). At 35 DAS, the treatment receiving P nine days earlier (P + 26DAS) showed a significantly increased P uptake, with values just around the sufficiency level at 2000 μg P g^{-1} DW, relative to the non P fertilized treatments, but with no clear differences in the growth stage development (Table 3). At 46 DAS, the P fertilized treatments had all entered the tillering stage and showed significantly larger P concentrations in YFEL compared to the non P fertilized treatments. The P + 35DAS treatment was still below the critical threshold limit at 2000 μg P g^{-1} DW, but apparently it was sufficient to enter the next growth stage without a reduction in final grain yields. At 56 DAS, P deficiency suddenly disappeared as all six treatments showed values above 2000 μg P g^{-1} DW, including for the P treatment, which did not receive any P throughout the experiment. This effect was also observed 69 DAS. The cause for the sudden increase in tissue P concentration for the non P fertilized treatments at 56DAS (treatment P + 56DAS and P-; Fig. 2) is not clear, but could be caused by either (i) remobilization of P from the root system or from older source leaves to the younger sink leaves, due to the change in growth strategy as described above, (ii) a sudden release of P from the soil or sufficient development of roots to explore P in the deeper root layers or (iii) significant P contribution from symbiosis with arbuscular mycorrhizal fungi. Furthermore, the leaf P concentration of annual plants generally declines with time as leaves undergo sink-source transition during aging (Grant et al. 2001), which was confirmed in this study, as the P tissue concentration for P + 0DAS went from 3000 to 2000 μg P g^{-1} DW from 26 to 69 DAS (Fig. 2).

The present study underlines the importance of timing when performing P analysis of leaf tissue. Despite clear visual and developmental differences between P + 0DAS and P- at 56 DAS (Fig. 1; Table 3), analysis of the P concentration in the YFEL was not able to differentiate between the two treatments as both treatments showed values >2000 μg P g^{-1} DW (Fig. 2). However, only ten days earlier (46 DAS) significant differences were observed between P + 0DAS and P- with values >3000 and < 1000 μg P g^{-1} DW, respectively. During the ten days from 46 to 56 DAS, the barley plants most likely changed growth strategy, causing a remobilization of the mobile nutrients (including P) within the plant (White and Veneklaas 2012) to prioritize and fully supply the tillers being maintained with P. Thus, it is important to perform diagnostic P analysis during early vegetative growth (before 46 DAS in the present experiment), as the obtained results might reflect P remobilization within the plant rather than the overall plant P status.

The clear correlations between P tissue concentration in YFEL and the appearance of the I-step obtained in this study confirms that chlorophyll *a* fluorescence transients is a valuable tool for diagnosing P deficiency directly in the field. However, to avoid yield loss, it is important to measure the P status of barley early in the growth season as plants are irreversibly damaged by P limitation during this period (Zadoks stage 12–15). Low P availability during early vegetative growth will severely and irreversibly decrease tiller formation and result in significant lower yields. In the present field experiment, P deficient conditions could be corrected to overcome a potential yield reduction if P was applied no later than 35 DAS. Phosphorus application after 35 DAS was unable to restore the induced metabolic disturbances, resulting in a significant reduction in grain yield. During prolonged P deficiency, P will be remobilized within the plant to support the existing tillers, and P tissue analysis performed too late in the vegetative growth phase (after 46 DAS in the present study) will not reflect the true demand of P. The soil used in this study generally represents a typical Scandinavian agricultural soil, and it is still unclear if the results can be transferred to other soil conditions. To further evaluate the power of chlorophyll *a* fluorescence to detect P deficiency in crops, future studies need to include other important crops,

cultivated across different soil types under a wider range of climatic conditions.

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Compliance with ethical standards

Conflict of interest A.C., J.F., and S.H. are co-applicants on the patent (PCT/EP2013/069899) describing the use of chlorophyll *a* fluorescence to determine the nutritional status of plants, and are founders of the university spin-out SpectraCrop IVS, marketing a hand-held device for predicting the P status of plants. For this reason, there may be a conflict of interest in order to show that the developed P model is able to predict the P status of barley leaves under field conditions. It should be noted that similar OJIP transients, as the ones recorded in this study, can be obtained by a broad range of standard chlorophyll *a* fluorescence instruments, including Photon Systems Instruments (PSI), Walz, Hansatech, Opti Sciences, Phenospex and MultispeQ.

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