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Research article

Alternate wetting and drying irrigation increases water and phosphorus use efficiency independent of substrate phosphorus status of vegetative rice plants

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ABSTRACT

Sustainable approaches to rice cultivation that apply less irrigation and chemical fertilisers are required to increase crop resource use efficiency. Although alternate wetting and drying (AWD) has been widely promoted as a water-saving irrigation technique, its interactions with phosphorus (P) nutrition have attracted little attention. Vegetative rice plants were grown with two phosphorus levels, fertilised (HP) *or* un-fertilised (LP), and either continuous flooding (CF) *or* AWD irrigation. Treatment effects on substrate P bioavailability (measured by Diffusive Gradients in Thin films – DGT-P), plant and substrate water relations, and foliar phytohormone status, were assessed along with P partitioning *in planta*. Shoot biomass and leaf area under different irrigation treatments depended on substrate P status (significant P x irrigation interaction), since LP decreased these variables under CF, but had no significant effect on plants grown under AWD. AWD maintained DGT-P concentrations and increased maximal root length, but decreased root P concentrations and P offtake. Substrate drying decreased stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}) but re-flooding increased g_s. AWD increased foliar abscisic acid (ABA), isopentenyl adenine (iP) and 1-aminocyclopropane-1-carboxylic acid (ACC) concentrations, but decreased *trans*-zeatin (tZ) and gibberellin A1 (GA1) concentrations. Low P increased ACC and jasmonic acid (JA) concentrations but decreased gibberellin A4 (GA4) concentrations. Across all treatments, stomatal conductance was negatively correlated with foliar ABA concentration but positively correlated with GA1 concentration. Changes in shoot phytohormone concentrations were associated with increased water and phosphorus use efficiency (WUE and PUE) of vegetative rice plants grown under AWD.

1. Introduction

Rice (*Oryza sativa* L.) is essential to global food security, but the increasingly unsustainable use of water and inappropriate use of limited nutrient resources means that new agronomic approaches are needed. Sustainable rice cultivation requires approaches that use less irrigation water and nutrient resources whilst maintaining (or improving) grain yields and nutritional quality.

Alternate wetting and drying (AWD) is an irrigation approach that repeatedly dries and re-floods fields, in contrast to continuous flooding (CF) rice cultivation. Irrigation is interrupted and the water table height allowed to decrease (due to drainage and/or crop evapotranspiration) until it reaches a certain level below the soil surface, after which the field is re-flooded. Although the agronomic effects of AWD vary with the duration and severity of soil drying, mild soil water deficits decreased water use by 23% while yields were statistically similar to continuously flooded crops, especially if AWD was applied either during the vegetative growth phase or reproductive growth phase, but not both (Carrijo et al., 2017). Nevertheless, this meta-analysis conceals considerable variation in the agronomic responses at specific sites, such

Abbreviations: AWD, alternate wetting and drying; CF, continuous flooding; DGT, Diffusive Gradients in Thin films; DW, dry weight; ET, evapotranspiration; FW, fresh weight; g_s, stomatal conductance; HP, fertilised with phosphorus; LP, not fertilised with phosphorus; MRL, maximal root length; Pi, inorganic orthophosphate; PUE, phosphorus use efficiency; WUE, water use efficiency; Ψ_{leaf}, leaf water potential

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that AWD sometimes significantly increases crop yields even though less water was applied (Mote et al., 2017; Norton et al., 2017a; Song et al., 2019). More detailed measurements of crop physiology are required to understand how AWD improves yield, but mechanisms may include leaf angle changes that allow greater light penetration of the canopy thereby boosting photosynthesis (Price et al., 2013), increasing the proportion of productive tillers (Howell et al., 2015; Mote et al., 2017; Norton et al., 2017a; Yang et al., 2017) and other grain yield components (Rahman and Bulbul, 2014; Li et al., 2018a; Song et al., 2019). AWD can be applied during grain filling to stimulate the remobilisation of stem carbohydrates that can contribute up to 40% of grain mass accumulation (Yang and Zhang, 2010). Since rice reproductive development can be sensitive to water deficits, AWD is sometimes applied only during the vegetative phase (Carrijo et al., 2017). Moreover, since the physiological responses of young rice plants have attracted little attention, our studies focused on vegetative rice plants as a model system.

With some exceptions, temporal dynamics of rice physiological responses such as stomatal conductance (Zhang et al., 2012) and leaf expansion (Howell et al., 2015; Norton et al., 2017a) during wetting and drying cycles has been little studied, although these processes may be regulated by root-to-shoot signalling of phytohormones (Yang et al., 2012). Dynamic changes in soil oxygen concentrations and soil matric potential during flooding and drying in AWD should affect root phytohormone synthesis and their export to the shoot. Each of 1 aminocyclopropane-1-carboxylic acid (ACC - the ethylene precursor), ABA and cytokinins are predicted to be uniquely related to soil water (or oxygen) status (Price et al., 2013). Applying AWD increased foliar ABA concentration relative to CF plants after several drying cycles, and increased foliar iP concentrations while decreasing leaf tZ concentrations (Norton et al., 2017a). In some experiments, treatment-induced changes in foliar phytohormone concentrations were stable despite fluctuating soil moisture conditions (Zhang et al., 2010; Song et al., 2019), yet in others significant effects on phytohormone concentrations were only detected at certain stages of the drying/re-flooding cycles (Norton et al., 2017a). Most hormone quantifications in rice plants responding to AWD used immunological approaches to detect specific hormones (Song et al., 2019) in the grains (Zhang et al., 2010, 2012). Alternatively, we utilised modern multi-analyte physico-chemical techniques (Todaka et al., 2017) to provide a more comprehensive analysis of phytohormone dynamics during AWD and its possible regulation of physiological responses (such as leaf growth and stomatal conductance) in vegetative rice plants.

There are increasing concerns about the future availability of mineral P to sustain global crop production (Sattari et al., 2012; Cordell and White, 2015; Blackwell et al., 2019), with inadequate P availability limiting plant growth, development and yield (Raghothama and Karthikeyan, 2005). Plants utilise inorganic orthophosphate (Pi) which can be of low availability and mobility in many soils. Multiple biological, chemical and physical mechanisms can interact with different forms of P in certain soil types, thereby affecting soil P availability during AWD. The anaerobic reducing conditions of flooded soils may release P from the organic fraction via redox-sensitive dissociation from iron/manganese oxides (Amery and Smolders, 2012). However, drying and re-flooding cycles such as occur during AWD can release P by physical (slaking of soil particles, colloidal detachment) or biological (soil microbial turnover due to desiccation upon drying and lysis upon re-watering) processes (reviewed in Dodd et al., 2015). AWD approximately doubled soil available P over 30 days, by stimulating the abundance of aerobic, putatively phosphate solubilising bacteria (Li et al., 2018a). While low P fertiliser addition rates magnify the effects of AWD on soil P release, different P fractions show unique responses, with AWD increasing $NaHCO_3$-Pi concentrations but decreasing HCl–P concentrations (Xu et al., 2020). Nevertheless, AWD decreased grain and straw P concentrations compared to continuous flooding (Ye et al., 2014; Norton et al., 2017b) and the response of shoot P content to AWD depends on the P fertiliser addition rate (Song et al., 2019; Zhang et al., 2019). Rice growth during the vegetative phase depends on root P absorption from the soil, with P remobilisation during the reproductive phase (Veneklaas et al., 2012). However, it is also important to understand P partitioning within the plant during the vegetative phase, especially as the root P concentrations are ignored in most studies (Cao et al., 2020).

While the effects of AWD, P and both factors applied separately on rice yield have been independently examined (Norton et al., 2017a, b; Yugandhar et al., 2017), their interactive effects on rice yields and leaf physiology have only attracted recent attention (Song et al., 2019; Zhang et al., 2019; Xu et al., 2020). Although the irrigation *x* P interaction did not always affect rice yields, plant hormonal responses can show complex interactions between soil water and P status (reviewed in Kudoyarova et al., 2015). Stomata of P-deficient cotton plants showed greater sensitivity to xylem-supplied ABA and closed at higher leaf water potentials (Ψ_{leaf}) in response to drying soil, and these plants accumulated more ABA at a given Ψ_{leaf} (Radin and Eidenbock, 1984). In contrast, P deficiency and osmotic stress had opposing effects on ABA accumulation in tomato while synergistically enhancing stomatal closure when both stresses co-occurred (Li et al., 2018b). Moreover, low P conditions influence *in planta* concentrations of multiple phytohormones (Rouached et al., 2010), with low P decreasing foliar GA, IAA and ZR contents (by 26, 13% and 22% respectively) of rice at the grain filling stage even though there was no interaction with AWD irrigation (Song et al., 2019). Whether AWD interacts with P status to regulate rice leaf phytohormone concentrations during the vegetative phase does not appear to have been studied.

Although many rice-growing regions have low P soil (Kekulandara et al., 2019; Xu et al., 2019) and field experiments have evaluated plant responses to different nutrient and water management practices (De Bauw et al., 2019a; Zhang et al., 2019), further work is needed to understand phytohormone responses to low P conditions and their physiological significance in vegetative plants grown under contrasting substrate water availabilities. Furthermore, most tests of soil P bioavailability chemically extract P from potentially non-plant available pools and may not be suitable for all soil types (Moody et al., 2013). Here, a well-tested *in situ* dynamic technique DGT (Diffusive Gradients in Thin films) was used as a mechanistic surrogate of plant-available P to quantify P bioavailability (Zhang and Davison, 1995; Zhang et al., 1998). DGT accurately reflects both crop yield (Mason et al., 2010) and tissue concentration (Mundus et al., 2017) responses to P concentration and was preferred as an *in situ* measurement of P availability to rice plants (Six et al., 2012, 2013). In contrast, traditional tests (i.e. Olsen P) require removal and air drying of soil samples before extraction and analysis, possibly confounding any changes in P availability caused by AWD. The ability to deploy DGT devices directly into the soil negates this issue, allowing real time, non-destructive measurements of P availability.

Since AWD had contrasting effects on bioavailable substrate P and plant P concentration in different studies as indicated above, we firstly determined if low availability of phosphorus and AWD affected substrate phosphorus concentration and total P offtake by the plant. Secondly, we analysed whether plant physiological responses (vegetative growth and stomatal conductance) were related to leaf hormone and water relations. Therefore rice plants were grown in pots under controlled environment conditions and exposed to a factorial combination of irrigation (AWD *versus* CF) and P fertiliser (HP *versus* LP) treatments. We hypothesised that adaptive physiological responses to substrate water and P deficits mitigated the impact of either stress on plant vegetative growth.

2. Materials and methods

2.1. Substrate preparation

A growing substrate containing equal parts of a sandy loam field soil (Myerscough College, UK), horticultural sand (DA 16/30, Sibelco, UK) and peat (Evergreen Irish Moss Peat, Henry Altys Ltd, UK) was constructed by homogenizing in a cement mixer for 5 min, and passing

through a sieve with a 10 mm pore diameter. All the substrate received ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3) and magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) at a rate of 98 mg kg^{-1} or 0.49 g per pot; 69 mg kg^{-1} or 0.34 g per pot and 66 mg kg^{-1} or 0.33 g per pot, respectively. Based on the pot dimensions (18 cm diameter, 20 cm high – 5 L volume), these rates corresponded to 150 kg ha^{-1} N and 100 kg ha^{-1} of K, Mg and S. Half of the substrate received no additional P fertiliser (LP) while the other half received superphosphate fertilizer (HP) at a rate of 148 mg kg^{-1} or 0.75 g per pot that corresponded to 100 kg ha^{-1} P. Twenty pots were filled with each substrate.

2.2. Analysis of substrate phosphorus concentrations

Available phosphorus by DGT (DGT-P) was measured as previously described (Zhang et al., 1998). Briefly, a 0.08 cm diffusive gel and a 0.04 cm precipitated ferrihydrite gel for binding P (Santner et al., 2010) were prepared and assembled with a filter membrane in DGT samplers with a 3.14 cm^2 sampling window. DGT devices (supplied by DGT Research Ltd, Lancaster, UK) were deployed directly by gently pressing into the surface of the substrate 24 h after re-flooding the pots, and left *in situ* for 24 h. To determine DGT-P concentrations the Fe-oxide gel was recovered from the deployed DGT device and eluted overnight in 2 mL (V_{acid}) of 0.25 M H_2SO_4. Spectrophotometry measured eluate P concentration using the phosphomolybdenum blue method, allowing the mass (M) of P accumulated in the Fe-oxide gel to be calculated using the following equation:

$$M = C_e (V_{acid} + V_{gel})/fe. \quad (1)$$

where C_e is the concentration of phosphorus in the acid; V_{gel} is the volume of the Fe-oxide gel, 0.20 mL; *fe* is the elution factor for P (equalling 1).

The concentration of P measured by DGT (C_{DGT}) was calculated using the following equation:

$$C_{DGT} = M \cdot \Delta g/(D \cdot t \cdot A) \quad (2)$$

where Δg is the thickness of the diffusive gel (0.08 cm) plus the thickness of the filter membrane (0.014 cm), D is the diffusion coefficient of phosphate in the gel (based on temperature during deployment), t is deployment time and A is exposure area (A = 3.14 cm^2). The D values can be found on DGT Research website (www.dgtreserach.com).

Olsen P was determined by spectrophotometry of sodium bicarbonate extracts (Olsen et al., 1954) where absorbance readings of the eluent at 880 nm are plotted against standards of known P concentration. Olsen P concentrations were calculated in triplicate at the beginning of the trial, before applying either type of irrigation treatment. Table 1 summarises substrate P levels.

2.3. Plant material and experimental conditions

Rice (*Oryza sativa* L.cv. Kaybonnet) seeds were sown in 98 compartment (300 × 210 mm) trays and germinated for 25 days in the unfertilised substrate used for this trial. Seedlings of uniform average size were transplanted (25 days after sowing) to 5 L pots. Rice plants were grown in a naturally lit glasshouse with supplementary lighting providing a 13 h photoperiod when light levels dropped below 400 μmol m^{-2} s^{-1}. Day and night temperatures were 29 °C and 19 °C respectively while corresponding relative humidity were 36% and 49% respectively and [CO_2] averaged 457 ppm. The glasshouse is located at the Lancaster Environment Centre: 54° 2′ 49.2036" N and 2° 48′ 3.6000" W". The treatments comprised a 2 [P level: fertilised-HP *versus* unfertilised-LP] x 2 [types of irrigation: continuous flooding (CF) *versus* alternate wetting and drying (AWD)] factorial experiment. Each treatment had ten replicates, arranged in a randomized design. The experiment was conducted from 15 December 2016 (seeds imbibed) to 1 March 2017 (harvest date).

Table 1
Substrate P concentration measured by Diffusive Gradients in Thin films (DGT-P) and Olsen at the beginning of the experiment in unfertilised (LP) and fertilised (HP) treatments. Data for DGT-P and Olsen are means ± SE of 10 and 3 replicates respectively, with P Values reported.

Measurement	Unfertilised	Fertilised	Fold difference	P Value
DGT-P (μg l^{-1})	24.8 ± 1.3	41.2 ± 1.6	+66%	< **0.001*****
Olsen P (mg kg^{-1})	25.9 ± 0.5	33.2 ± 0.4	+28%	< **0.01****

For each P treatment, half the plants were continuously flooded (CF) with the water table maintained ~ 3 cm above the substrate surface by frequent irrigation to replace evapotranspirational losses. Throughout the experiment, changes in pot weight were recorded with a balance (Model CCEU20, Adam, Milton Keynes, UK) to determine crop water use (evapotranspiration). In addition, a measuring cylinder was used to record irrigation volume each time water had to be replaced. All pots included PVC pipes (25 cm high and 4 cm diameter, perforated with 1 cm diameter holes and covered with a mesh to avoid substrate entry) placed at the pot's edge (to minimise perturbation of root growth). These allowed water table height to be measured daily with a ruler. Individual plants dried the substrate at different rates, but all plants in the AWD treatment were re-flooded when the average water table height (across all plants) reached 15 cm below the substrate surface, following IRRI's guideline of "safe" AWD irrigation.

During the first 12 days after transplanting, all treatments were continuously flooded (CF) to allow plant establishment. During the next 12 days after transplanting all treatments were irrigated intermittently but the pots were not weighed. From 25 to 54 days after transplanting, plants were continuously flooded (CF) or received alternate wetting and drying (AWD).

2.4. Physiological measurements

A porometer (Model AP4, Delta-T Devices, Cambridge, UK) recorded stomatal conductance (g_s) daily, from the 26th day after transplanting. Measurements were made between 11:00h and 15:00h on the abaxial leaf surface of an upper canopy leaf.

Evapotranspiration (ET) was measured gravimetrically daily from the 26th day after transplanting based on the pot weight difference between consecutive days, allowing for any irrigation volume applied. Summing the individual values through the experiment allowed the accumulated evapotranspiration to be calculated.

A pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA) was used to measure leaf water potential (Ψ_{leaf}) at the end of the trial. Stomatal conductance had previously been measured in each leaf, which was excised, placed in the chamber immediately after collection and pressurised at a rate of 0.02 MPa s^{-1} until reaching the balancing pressure.

2.5. Phytohormone analysis

The same leaves used to measure stomatal conductance were sampled before (BR) and after re-flooding (AR) in the last cycle (54 days after transplanting) to measure ABA and other hormones according to a method based on Albacete et al. (2008). Cytokinins (*trans*-zeatin, tZ, zeatin riboside, ZR and isopentenyl adenine, iP), gibberellins (GA1, GA3 and GA4), indole-3-acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed. Leaves were immediately frozen in liquid nitrogen after excision, and then stored at −80 °C. Then, samples were freeze-dried and ground to a coarse powder using dissecting scissors. To prepare plant tissue for analysis, the samples were dropped in 1 mL of cold (−20 °C) extraction mixture of methanol/water (80/20, v/v). Centrifugation (20 000 × *g*, 15 min) separated the solids, which were re-extracted for 30 min at 4 °C in an additional 1 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak

Plus †C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40 °C under vacuum either to near dryness or until organic solvent is removed. The residue was dissolved in 1 mL of methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 μm pore size nylon membrane (Millipore, Bedford, MA, USA).

Ten μL of filtrated extract were injected in a U-HPLC-MS system comprising an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). To quantify the plant hormones, calibration curves were constructed for each analyte (1, 10, 50, and 100 μg L^{-1}) and corrected for 10 μg L^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%

2.6. Growth measurements

Leaf length was measured daily with a ruler, on 5 actively growing leaves per plant. Leaf relative growth rate was calculated at the end of the experiment using the following formula: Ln (Total leaf length at the end of the trial) - Ln (Total leaf length at the beginning of the trial)/ Elapsed time. A leaf area meter (Model 3100, Li-Cor, Lincoln, NE, USA) determined leaf area at the end of the experiment (54 days after transplanting, after 4 drying/re-flooding cycles).

At harvest, the substrate was gently washed from the roots and each plant divided into aerial parts (shoots) and roots to determine their fresh weight (FW). Maximal root length was measured with a ruler after carefully separating them from the substrate at the end of the trial. The number of tillers was counted also at the end of the trial. Then, shoots and roots were oven-dried at 80 °C for 1 week until they reached a constant mass to measure their respective dry weights (DW). Water-use efficiency (WUE) was calculated at the end of the trial for each plant as shoot DW divided by water used (accumulated evapotranspiration).

2.7. Nutrient analysis

After grinding dried root and shoot samples to a fine powder with a ball mill (Retsch MM400, Retsch UK Limited, Castleford, West Yorkshire, UK), they were subjected to microwave-assisted acid digestion (Mars-5 Xpress microwave-accelerated reaction system, CEM corporation, Matthews, NC, USA) in trace metal grade HNO_3 (Sigma-Aldrich, Dorset, UK) for 30 min at a maximum temperature of 200 °C. The digestate was then diluted to a final 2% (v/v) HNO_3 concentration with Millipore water and filtered through a 0.45 μm syringe filter. Inductively coupled plasma-optical emission spectrometry (ICP-OES; iCAP 6300, Thermo Scientific, MA, USA) was used to analyse P concentrations by comparing against standards of a known range of concentrations, and corrected, if required, using determinations from blank HNO_3 samples run in the microwave digestion. Concentrations of P (for both shoot and root) were expressed in mg g^{-1} DW and tissue P content calculated by multiplying tissue concentration by the total dry weight of each respective component at the end of the trial. Shoot and root P contents were summed to calculate total plant P offtake at the end of the trial. Phosphorus-use efficiency (PUE) was calculated at the end of the trial for each plant as total DW divided by total P offtake.

2.8. Statistical analysis

Significant differences in substrate P concentration between HP and LP treatments were determined using Student's t-test. Three-way-ANOVA determined if re-flooding (before and after) and its interaction with other factors (irrigation and phosphorus) significantly affected plant response (Supplementary Table 1). If there was no significant

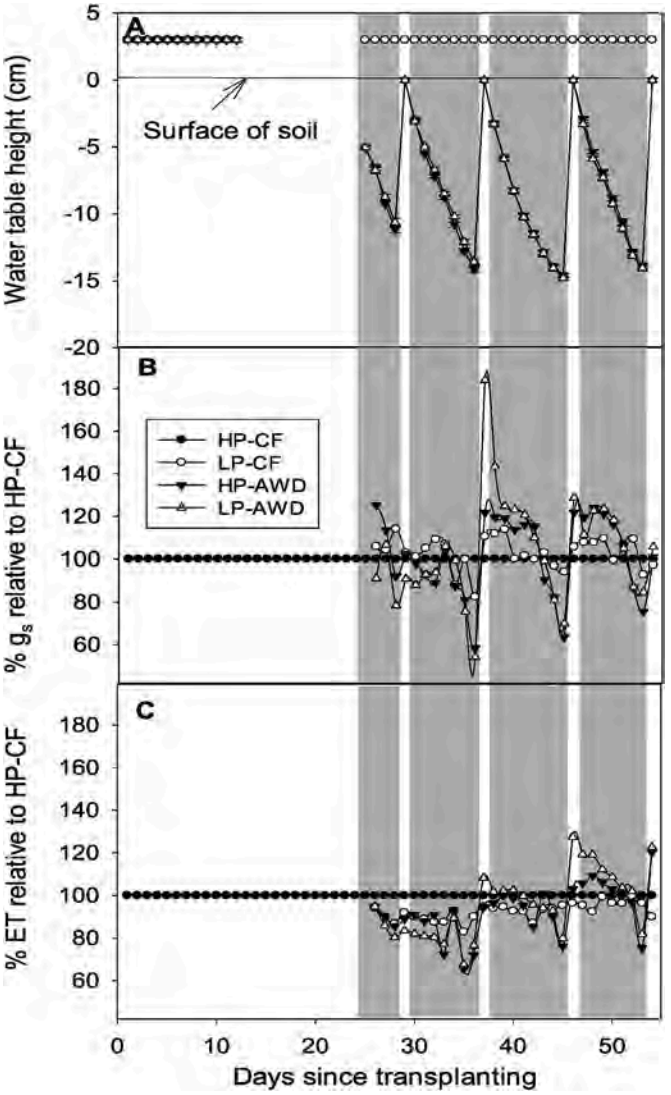

Fig. 1. (A) Water table height, (B) relative stomatal conductance (g_s) and (C) relative evapotranspiration (ET) measured since transplanting. Shading indicates periods of intentional substrate drying. HP = fertilised with phosphorus, LP = un-fertilised with phosphorus, CF = Continuous flooding and AWD = Alternate wetting and drying. Between 13 and 24 days since transplanting, water table height was not monitored and plants were watered intermittently. Relative g_s and ET were expressed as percentage of the HP-CF control, which maintained an average g_s of 311 mmol m^{-2} s^{-1}. Data are means of 10 replicates. Error bars omitted for clarity.

effect of re-flooding, the data before and after-re-flooding were pooled for analysis by two-way-ANOVA to discriminate significant effects of substrate P status, irrigation and their interaction. Treatment means were separated with Lincoln's Multiple Comparisons (Robust Test) to determine statistical differences. Correlations between different variables were established using a LTS (least trimmed squares) procedure, as described in correlation matrixes (Tables 4 and 5) and specific figures (Figs. 2 and 3). All statistical analyses were carried out with R version 3.5.3 (2019-03-11) Copyright (C) 2019 The R Foundation for Statistical Computing Platform: i386-w64-mingw32/i386 (32-bit).

3. Results

The water table of the CF plants was maintained 3 cm above the substrate surface, while that of the AWD plants declined to 15 cm before re-flooding (Fig. 1a). Four drying and re-flooding cycles were

Table 2
Leaf water potential (Ψ_{leaf}) before re-flooding and accumulated evapotranspiration (ET) of plants grown under high (HP) and low phosphorus (LP) and alternate wetting and drying (AWD) and continuous flooding (CF). Data are means ± SE of 10 replicates with different letters for each row indicating significant differences ($P < 0.05$) as determined by robust Lincoln's multiple comparisons test.

	HP-CF	LP-CF	HP-AWD	LP-AWD	Phosphorus (P)	Irrigation (I)	P *x* I
Ψ_{leaf} (MPa)	−1.30 ± 0.02 a	−1.32 ± 0.02 ab	−1.37 ± 0.03 b	−1.39 ± 0.02 b	0.46 n.s.	0.009**	0.37 n.s.
Water used (L) (accumulated ET)	6.8 ± 0.2 a	6.3 ± 0.2 ab	6.0 ± 0.1 b	6.0 ± 0.2 b	0.35 n.s.	0.007**	0.72 n.s.

Two-way ANOVA results (P-values reported). Treatment effects are: not significant (n.s., $P > 0.05$) and $P \leq 0.01$ (**).

Table 3
Foliar hormone concentrations (expressed in ng g^{-1} DW) measured at the end of the trial (last cycle) of plants grown under high (HP) and low phosphorus (LP) and alternate wetting and drying (AWD) and continuous flooding (CF). ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; GA, gibberellin; iP, isopentenyl adenine; JA, jasmonic acid; tZ, *trans*-zeatin. Data are means ± SE of 10 replicates with different letters for each row indicate significant differences ($P < 0.05$) as determined by Lincoln's multiple comparisons robust test.

	HP-CF	LP-CF	HP-AWD	LP-AWD	Phosphorus (P)	Irrigation (I)	P *x* I
ABA	26.0 ± 2.1b	30.7 ± 1.5b	37.3 ± 2.0 a	40.4 ± 1.1 a	0.10 n.s	0.004**	0.41 n.s
tZ	1825 ± 53 ab	2317 ± 189 a	1628 ± 156 b	1638 ± 119 b	0.07 n.s	0.007**	0.030*
iP	19.2 ± 1.6 ab	14.1 ± 2.1 b	19.8 ± 2.6 ab	21.1 ± 1.2 a	0.50 n.s	0.045*	0.039*
JA	67 ± 9 ab	80 ± 9 a	59 ± 4 b	88 ± 6 a	0.014*	0.61 n.s	0.63 n.s
ACC	79 ± 26 b	337 ± 59 a	118 ± 13 b	484 ± 61 a	< 0.001***	0.046*	0.24 n.s
GA1	0.60 ± 0.13 a	0.55 ± 0.15 a	0.18 ± 0.07 b	0.24 ± 0.10 b	0.95 n.s	0.016*	0.66 n.s
GA4	1.66 ± 0.36 a	0.43 ± 0.07 b	1.30 ± 0.52 a	0.58 ± 0.16 b	0.005**	0.74 n.s	0.44 n.s

Two-way ANOVA results (P-values reported). Treatment effects are: not significant (n.s., $P > 0.05$), $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P < 0.001$ (***).

applied, differing in duration between 4 (the initial cycle which started when the substrate had already dried) and 8 days of substrate drying.

3.1. Physiological measurements

Changes in substrate water dynamics altered stomatal conductance (g_s) that to some extent explained changes in evapotranspiration (cf. Fig. 1b and c). In the HP-CF plants, g_s fluctuated considerably throughout the experiment (200–900 mmol m^{-2} s^{-1}); possibly caused by plant development and environmental conditions (fluctuating light intensity and VPD). Thus, values for the other treatments were expressed as percentages of these HP-CF plants (Fig. 1b and c). AWD caused pronounced oscillations in g_s, especially during the last two cycles, with lower values before re-flooding (BR) and higher values after re-flooding (AR). After re-flooding at the end of the trial, g_s of all plants was similar (Fig. 1b). When leaf water potential (Ψ_{leaf}) was measured at the end of the final drying cycle, plants exposed to drying substrate had a lower Ψ_{leaf} (by 0.07 MPa) than the continuously flooded plants (Table 2), but P level did not affect Ψ_{leaf}. Similarly, accumulated evapotranspiration was 8% lower in AWD plants than CF plants.

3.2. Foliar hormone analysis

Since re-flooding had no significant effects on foliar hormone concentrations at the end of the last drying cycle, data were pooled (before and after re-flooding, Supplementary Table 1). Foliar ABA concentration was 37% higher (averaged across P treatments) in the AWD treatments than the CF treatments (Table 3). AWD caused contrasting changes in the foliar concentrations of the two cytokinins measured: [*trans*-zeatin, tZ] and [isopentenyl-adenine, iP] (Table 3). For both cytokinins, substrate P status affected the response to AWD treatment (as indicated by significant irrigation *x* P treatment interactions). Under continuous flooding, the LP treatment tended to have higher tZ concentrations than the HP treatment, even though both AWD treatments had similar values (which were 20% lower than in CF plants, averaging across P treatments) (Table 3). Conversely, under continuous flooding, the LP treatment tended to have lower iP concentrations than the HP treatment, even though both AWD treatments had similar values (which were 26% higher than in CF plants, averaging across P treatments). Under LP, the AWD treatment increased leaf iP concentration by ~50%. Taken together, foliar cytokinin status was affected more by irrigation than substrate P status (Table 3).

Additionally, AWD also decreased leaf GA1 concentrations by 63% and increased leaf ACC concentrations by 46% averaged across P treatments (Table 3). The low P treatments also caused pronounced changes in ACC, GA4 and JA concentrations, largely independent of irrigation regime. LP increased ACC concentrations by 4-fold compared to the respective HP treatments averaged across irrigation treatments. Furthermore, low P increased JA concentrations by 34% and decreased GA4 concentrations by 65% compared to the high P plants (Table 3). Thus the concentrations of some phytohormones were primarily affected by irrigation treatment (ABA, GA1, iP and tZ) and others by P treatment (GA4, JA), while both factors affected ACC concentrations.

3.3. Correlation analysis

Before re-flooding during the last AWD cycle, across all treatments, stomatal conductance decreased as leaf ABA concentration increased (Fig. 2a, Table 4) and as Ψ_{leaf} decreased (Fig. 2b, Table 4). Furthermore, leaf ABA concentration increased as Ψ_{leaf} decreased (Fig. 2c, Table 4). When measurements before and after re-flooding were combined, stomatal conductance was still negatively correlated with leaf ABA concentration (Fig. 3a), but also positively correlated with leaf GA1 concentration (Fig. 3b). In contrast, g_s was not correlated with foliar concentrations of ACC, cytokinins (iP and tZ) and JA (Table 5).

When measurements before and after re-flooding were combined, shoot [P] was negatively correlated with leaf ABA (Fig. 3c) and ACC concentrations (Fig. 3d) but positively correlated with leaf tZ concentration (Table 5). Thus, leaf hormone concentrations were significantly correlated with shoot P status irrespective of substrate moisture dynamics.

Different hormone concentrations and growth variables were also correlated. Leaf ACC concentration was negatively correlated with leaf GA4 concentration, but positively correlated with leaf ABA concentration (Table 5). Leaf ABA concentration was negatively correlated with leaf GA1 concentration but positively correlated with leaf iP concentration. Finally, leaf iP concentration was negatively correlated with leaf GA1 concentration (Table 5). Moreover, shoot dry weight, leaf area and tiller number were positively correlated with leaf iP concentration. Leaf relative growth rate was positively correlated with leaf GA4 and

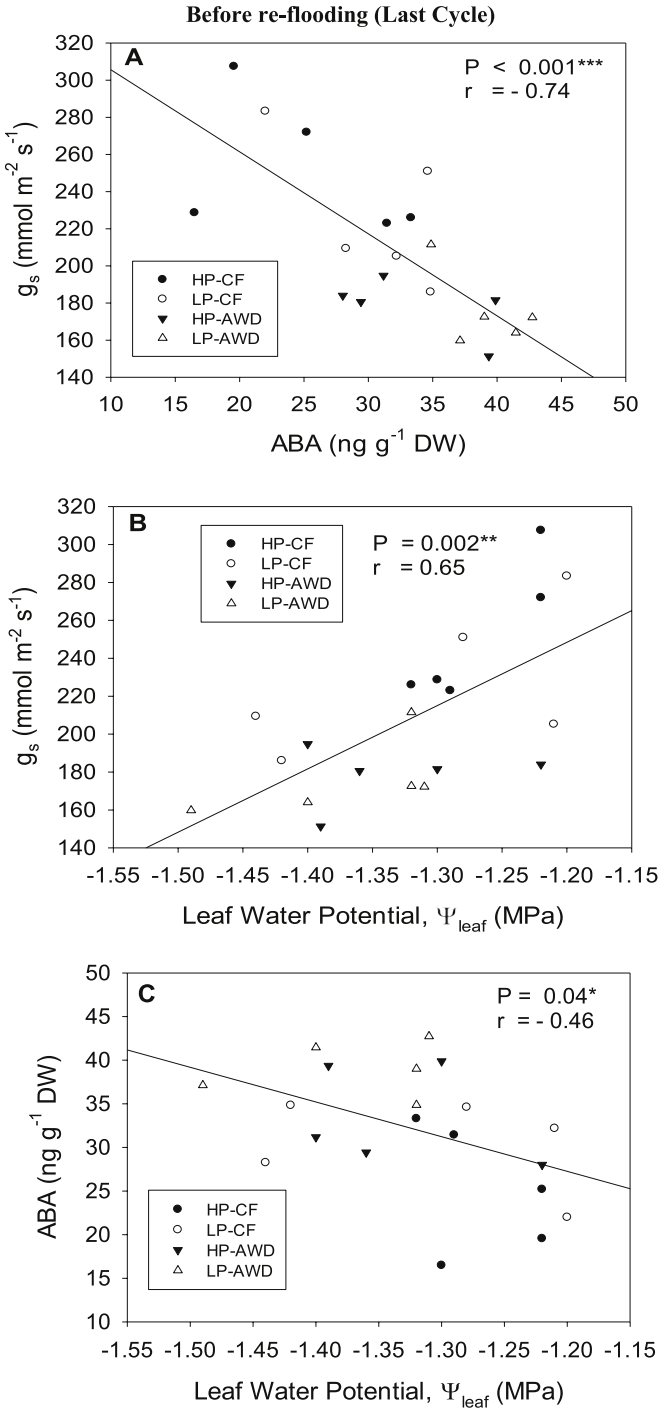

Fig. 2. (A) Stomatal conductance (g_s) plotted against foliar ABA concentration and (B) leaf water potential (Ψ_{leaf}) before re-flooding during the last drying cycle. (C) Foliar ABA concentration plotted against leaf water potential (Ψ_{leaf}) before re-flooding of last cycle. Symbols represent individual plants, with regression lines fitted where significant.

shoot P concentrations, and negatively with leaf ACC concentrations (Table 5).

3.4. Substrate P measurements and plant P analysis

Despite both DGT and Olsen measured P in the LP substrate being above reported critical P thresholds (the level at which 80% of maximum potential yield would be expected) for rice (Six et al., 2013). P fertiliser significantly increased shoot dry biomass and leaf area in the CF treatment (Table 7). This suggests P was limiting even though shoot tissue P at the end of the experiment was the same as the HP treatment (Table 6), probably because P fertiliser addition benefited early crop development (Grant et al., 2001).

Table 4
Correlation matrix among stomatal conductance (g_s), leaf water potential (Ψ_{leaf}) and foliar abscisic acid (ABA) concentration before re-flooding.

	g_s	Ψ_{leaf}	ABA
g_s	r = 1.00		
Ψ_{leaf}	r = 0.65 (**P = 0.002****)	r = 1.00	
Leaf [ABA]	r = −0.74 (**P < 0.001***)	r = −0.46 (**P = 0.04***)	r = 1.00

r = Pearson correlation coefficient and P values reported for significant correlations (bold text). Treatment effects are: P ≤ 0.05 (*), P ≤ 0.01 (**) and P < 0.001 (***).

At the beginning of the trial and after the first drying and re-flooding cycle, differences in DGT-P concentrations were determined solely by the initial P fertiliser application, with DGT-P concentrations of the HP treatments 71% higher than the LP treatments (Fig. 4). Thereafter, when DGT-P concentration was measured in re-flooded substrate, the AWD treatments had significantly higher DGT-P concentrations than their respective CF treatments (Fig. 4). At the final measurement, the HP-AWD and LP-AWD treatments had 52 and 80% higher DGT-P concentration than their respective CF treatments (Fig. 4).

Neither P nor irrigation treatment significantly affected shoot P concentration. AWD decreased root P concentration by 26% (averaged across both P treatments) (Table 6). Shoot and root P contents (multiplying tissue P concentrations by their respective biomass) showed similar patterns, with no significant effect of either treatment on shoot P content, while AWD decreased root P content (Table 6). Under continuous flooding, the low P treatment slightly decreased (by 11%) total P offtake compared to the high P treatment. AWD decreased total P offtake by 18% (averaged across both P treatments) (Table 6). These differences in total P offtake (biomass x concentration) may explain the differences in DGT-P between CF and AWD treatments. Although the decrease in DGT-P from the start to the end of the experiment (Delta DGT-P) was less in AWD (HP -6.82 and LP -5.23 μg L^{-1}) than CF (HP -12.23 and −10.27 μg L^{-1}), crop P offtake was also lower in the AWD plants (HP 40.4 and LP 39.9 mg) than the CF (HP 51.7 and 45.9 mg) plants (Fig. 5). Thus, the difference in DGT-P measured at the beginning and end of the experiment was related to total P offtake across all treatments (Table 6, Fig. 5).

3.5. Growth measurements

In the high P treatments, AWD had no significant effect on shoot DW, leaf area and tiller number compared to CF plants, but decreased leaf RGR by 2% (Table 7). In the low P treatments, AWD and CF treatments had the same number of tillers, but the AWD treatment increased shoot DW, leaf area and leaf RGR by 8%, 13% and 3% respectively compared to CF plants. Thus, the response of shoot DW and leaf area/RGR to AWD depended on P status (as indicated by significant irrigation *x* phosphorus interactions) (Table 7).

Below-ground, root dry weight and root/shoot ratio were similar across all P/irrigation treatments although the LP-AWD treatment had higher values (Table 7). Nevertheless, both irrigation and P treatment significantly affected maximal root length (MRL). Under continuous flooding, LP increased MRL by 37% compared to the HP treatment. Under HP conditions, AWD increased MRL by 49% compared to the CF treatment. These effects were additive such that LP-AWD plants had the longest MRL (Table 7). Thus, suboptimal resource supply stimulated root growth.

Finally, total DW was similar in the AWD treatments as indicated by

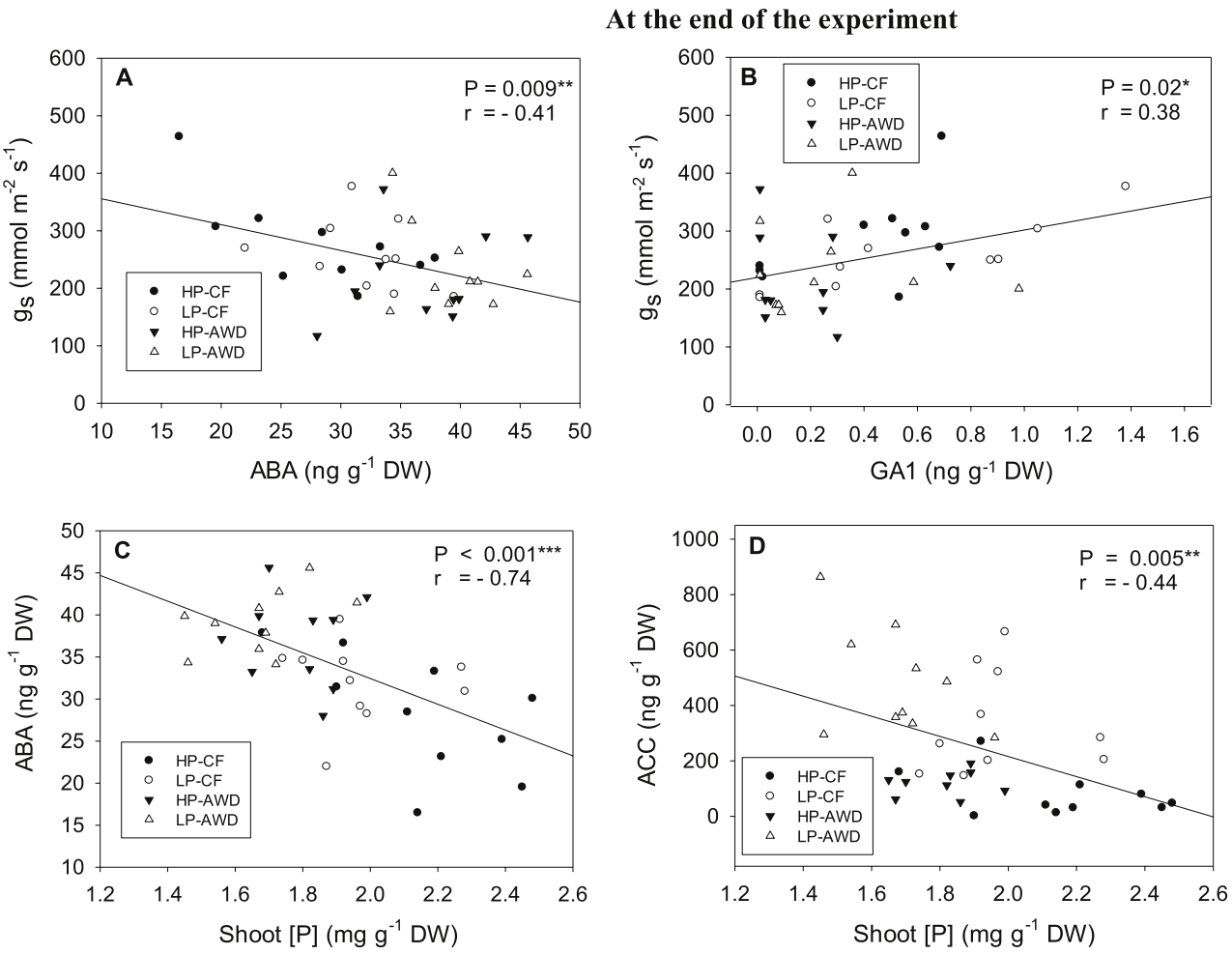

Fig. 3. (A) Stomatal conductance (g_s) plotted against foliar ABA concentration and (B) GA1 concentration before and after re-flooding at the end of the experiment. (C) Foliar ABA concentration plotted against shoot [P] concentration and (D) foliar ACC concentration plotted against shoot [P] concentration before and after re-flooding at the end of the experiment. Symbols represent individual plants, with regression lines fitted where significant.

a significant irrigation *x* phosphorus interaction. Under continuous flooding, low P decreased total DW by 8%. AWD increased water use efficiency (WUE = shoot dry weight/accumulated ET) by 9% (averaged across P treatments), but there was no significant effect of P treatment on WUE (Table 7). Similarly, AWD also increased phosphorus use efficiency (PUE = total dry weight/total P offtake) by 23% (averaged across P treatments), but there was no significant effect of P treatment (Table 7). Taken together, AWD significantly increased WUE and PUE compared with CF.

4. Discussion

Alternate wetting and drying irrigation can enhance rice water use efficiency without significantly diminishing yields (Price et al., 2013; Howell et al., 2015; Carrijo et al., 2017) and multiple soil incubation experiments demonstrate that soil drying and re-wetting can enhance soil P concentrations via different mechanisms (Forber et al., 2017). More recently, greenhouse (Song et al., 2018) and field (Song et al., 2019; Zhang et al., 2019; Xu et al., 2020) experiments have investigated interactions between AWD and P treatments on rice physiology and yield, but soil P dynamics were not always monitored (Song et al., 2018, 2019). For the first time, deploying DGT devices allowed *in situ* measurements of substrate solution P dynamics, demonstrating that AWD irrigation maintained available P concentrations over 54 days, whereas substrate P concentrations declined by approximately 40% in the CF treatments (Fig. 4). Recent field trials established that AWD can increase soil P concentrations (Xu et al., 2020) by increasing the proportion of aerobic bacteria (which participate in nutrient cycling) and enzyme activities such as phosphatase (Li et al., 2018a), and increasing the abundance of bacteria with acid phosphatase activity to release available P in the rhizosphere (Zhang et al., 2019). However, our plants grown with AWD captured less P resulting in lower P offtake than the CF treatments (Table 6), thus substrates exposed to AWD maintained higher DGT-P values than CF substrates at the end of the experiment (Figs. 4 and 5). Furthermore, multi-analyte hormone measurements demonstrated previously unreported effects, that AWD stimulated leaf ACC concentration, that low P enhanced leaf ACC and JA concentrations and that both factors interacted to determine leaf cytokinin (iP, ZR) concentrations. Foliar hormone concentrations were correlated with stomatal and shoot growth regulation, thereby increasing water and phosphorus use efficiency, especially under AWD irrigation.

Exposing rice plants to a factorial combination of phosphorus (P) fertiliser and irrigation treatments showed that AWD enhanced PUE (Table 7) independently of soil P status, suggesting that P allocation within the plants was also important in determining PUE. Although AWD irrigation maintained substrate P concentrations, shoot P concentrations were not changed and root P concentrations declined by 26% compared to CF irrigation (Table 6), indicating that enhanced P availability did not benefit plant nutrient uptake and growth (Fig. 4). Thus it is important to understand why AWD caused differences in P partitioning, resulting in similar shoot P concentrations to CF treatments (Table 6). Plants coordinate inorganic phosphate (Pi) acquisition

Table 5
Correlation matrix among stomatal conductance (g_s), shoot P concentration, shoot dry weight, leaf area, tillers, leaf relative growth rate (LRGR) and different foliar hormone concentrations at the end of the experiment. ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; GA, gibberellin; iP, isopentenyl adenine; JA, jasmonic acid; tZ, *trans*-zeatin.

	g_s	ABA	GA1	GA4	ACC	JA	tZ	iP	Shoot [P]	Shoot DW	Leaf area	Tillers
g_s	r = 1.00											
ABA	r = −0.41 **(P = 0.009**)**	r = 1.00										
GA1	r = 0.38 **(P = 0.015*)**	r = −0.38 **(P = 0.02*)**	r = 1.00									
GA4	r = −0.12	r = −0.22	r = −0.03	r = 1.00								
ACC	r = −0.19	r = 0.42 **(P = 0.009**)**	r = −0.06	r = −0.45 **(P = 0.003**)**	r = 1.00							
JA	r = 0.07	r = 0.10	r = −0.03	r = −0.27	r = 0.25	r = 1.00						
tZ	r = −0.09	r = −0.05	r = 0.27	r = −0.04	r = 0.22	r = 0.03	r = 1.00					
iP	r = −0.02	r = 0.41 **(P = 0.009**)**	r = −0.40 **(P = 0.012*)**	r = −0.03	r = 0.03	r = 0.18	r = −0.05	r = 1.00				
Shoot [P]	r = 0.22	r = −0.58 **(P < 0.001***)**	r = 0.25	r = 0.26	r = −0.43 **(P = 0.005**)**	r = −0.11	r = 0.40 **(P = 0.009**)**	r = −0.09	r = 1.00			
Shoot DW	r = 0.06	r = −0.06	r = −0.24	r = 0.05	r = −0.06	r = −0.11	r = −0.17	r = 0.31 **(P = 0.048*)**	r = 0.04	r = 1.00		
Leaf area	r = 0.04	r = 0.07	r = −0.19	r = 0.28	r = −0.22	r = −0.17	r = −0.20	r = 0.36 **(P = 0.024*)**	r = 0.05	r = 0.72 **(P < 0.001***)**	r = 1.00	
Tillers	r = 0.17	r = −0.08	r = −0.02	r = 0.12	r = −0.20	r = −0.01	r = 0.00	r = 0.33 **(P = 0.033*)**	r = 0.20	r = 0.63 **(P < 0.001***)**	r = 0.57 **(P < 0.001***)**	r = 1.00
LRGR	r = −0.10	r = −0.20	r = 0.00	r = 0.41 **(P = 0.009**)**	r = −0.36 **(P = 0.023*)**	r = −0.10	r = −0.28	r = 0.03	r = 0.32 **(P < 0.041*)**	r = 0.20	r = 0.22	r = 0.19

r = Pearson correlation coefficient and P values reported for significant correlations (bold text). Treatment effects are: P ≤ 0.05 (*), P ≤ 0.01 (**) and P < 0.001 (***).

Table 6
Tissue Phosphorus (P) concentrations (expressed in mg g^{-1} DW) at the end of the trial, along with phosphorus (P) content (multiplying P concentration by tissue dry weight) and total P offtake (summing root and shoot P contents) of plants grown under high (HP) and low phosphorus (LP) and continuous flooding (CF) and alternate wetting and drying (AWD). Data are means ± SE of 10 replicates with different letters for each row indicating significant differences ($P < 0.05$) as determined by robust Lincoln's multiple comparisons test.

	HP-CF	LP-CF	HP-AWD	LP-AWD	Phosphorus (P)	Irrigation (I)	P *x* I
Shoot [P]	2.04 ± 0.13 a	1.99 ± 0.09 a	1.85 ± 0.07 a	1.80 ± 0.11 a	0.53 n.s	0.07 n.s	0.25 n.s
Root [P]	1.59 ± 0.06 a	1.56 ± 0.08 a	1.20 ± 0.05 b	1.15 ± 0.05 b	0.77 n.s	< 0.001***	0.48 n.s
Shoot P content (mg plant^{-1})	22.6 ± 1.7 a	19.7 ± 1.3 a	19.6 ± 0.9 a	19.4 ± 1.5 a	0.14 n.s	0.12 n.s	0.52 n.s
Root P content (mg plant^{-1})	29.1 ± 2.6 a	26.3 ± 2.9 ab	20.8 ± 2.4 b	20.3 ± 2.4 b	0.41 n.s	0.0052**	0.50 n.s
Total P offtake (mg plant^{-1})	51.7 ± 2.6 a	45.9 ± 3.4 ab	40.4 ± 2.7 b	39.8 ± 3.2 b	0.26 n.s	0.0091**	0.39 n.s

Two-way ANOVA results (P-values reported). Treatment effects are: not significant (n.s., $P > 0.05$), $P \leq 0.01$ (**) and $P < 0.001$ (***).

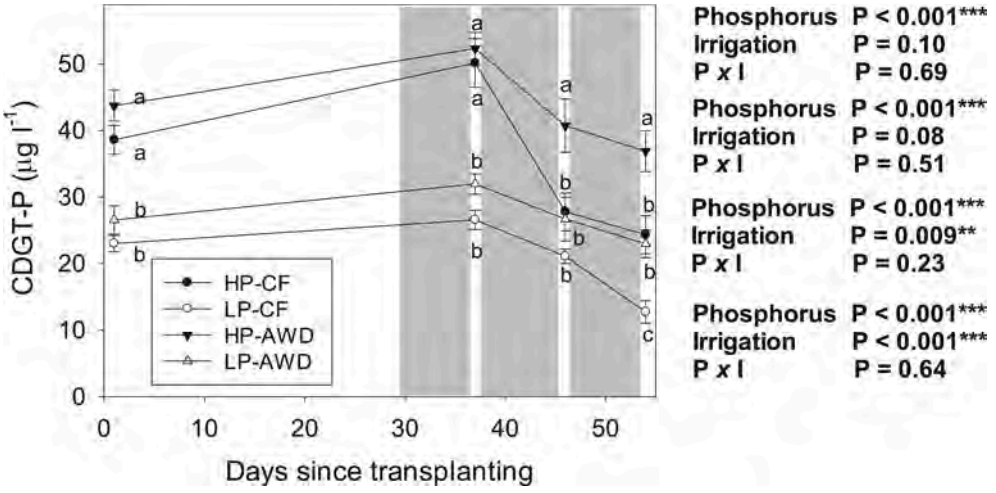

Fig. 4. Substrate phosphorus concentrations measured using Diffusive Gradient in Thin films (DGT) technique since transplanting. Shading indicates periods of intentional substrate drying. HP = fertilised with phosphorus, LP = un-fertilised with phosphorus, CF = Continuous flooding and AWD = Alternate wetting and drying. Data are means ± SE of 10 replicates with two-way ANOVA results (P-values reported). Different letters indicate significant differences ($P < 0.05$) as determined by robust Lincoln's multiple comparisons test. P Values for phosphorus (P), irrigation (I) and their interaction presented at right of the panel, from early (top) to late (base) in the experiment.

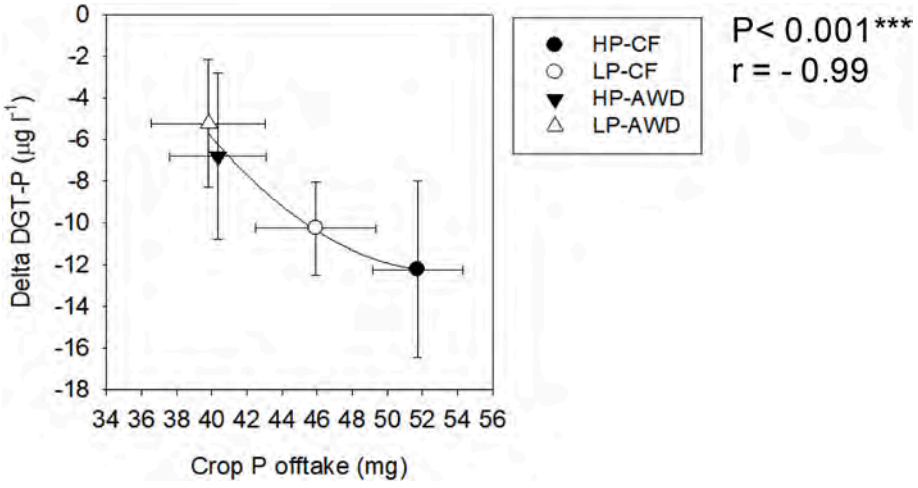

Fig. 5. Difference in DGT measured at the beginning and the end of the transplanting (Delta DGT-P) plotted against total crop P offtake, for plants grown under fertilised (HP) and un-fertilised (LP) with phosphorus and continuous flooding (CF) and alternate wetting and drying (AWD). Data are means ± SE of 10 replicates, with an exponential decay regression fitted to all points.

and translocation from root to shoots under P-deficient conditions via hormonal mechanisms. Cytokinins may promote root-to-shoot Pi translocation by modulating rice ortholog(s) of the Arabidopsis *PHO1* gene (Secco et al., 2010). Although *pho1* mutants were less sensitive to cytokinins, cytokinin treatments increased shoot Pi content of *pho1* mutants to WT levels. Thus interactions between phosphate signalling (under P-deficient conditions) and cytokinin signalling through the *PHO1* gene (Rouached et al., 2011) may explain some of the physiological responses to AWD. Since root and leaf cytokinin concentrations change similarly in response to AWD and low P (Zhang et al., 2010; Song et al., 2019), the high iP concentration in our LP-AWD treatment (Table 3) could favour P translocation from roots to the shoots.

Cytokinins may also promote vegetative growth. AWD mitigated the effects of low substrate P status on shoot growth (shoot DW, leaf area and tiller number), as indicated by significant irrigation *x* phosphorus interactions (Table 7). AWD decreased leaf [tZ] by 10–30% (according to P treatment) compared to CF plants (Table 3), as in field-grown plants (Norton et al., 2017a) but also increased leaf [iP] by 19–27%, possibly related to increased expression of *OsIPT* isopentenyltrasferase (Liu et al., 2011). Cytokinins may enhance leaf growth by regulating activity of expansin proteins thereby increasing cell wall extensibility (Downes and Crowell, 1998). Moreover, down-regulation of cytokinin oxidase 2 (*CKX2*) expression (Yeh et al., 2015) and overexpressing the *OsIPT2* or *OsIPT3* genes (Sakamoto et al., 2006) promoted cytokinin accumulation thereby enhancing tillering. However, greater responsiveness of tiller number (Table 7) than cytokinin levels (Table 3) to P treatment, and similar tiller numbers under LP conditions irrespective of irrigation treatment demonstrates a more complex regulation of tiller

Table 7
Plant growth, water use efficiency (WUE) and phosphorus use efficiency (PUE) measured at the end of trial, of plants grown under high (HP) and low phosphorus (LP) and alternate wetting and drying (AWD) and continuous flooding (CF). Data are means ± SE of 10 replicates with different letters for each row indicating significant differences ($P < 0.05$) as determined by robust Lincoln's multiple comparisons test.

	HP-CF	LP-CF	HP-AWD	LP-AWD	Phosphorus (P)	Irrigation (I)	P *x* I
Shoot Dry Weight (g plant^{-1})	11.4 ± 0.3 a	9.8 ± 0.4 b	10.40 ± 0.2 ab	10.57 ± 0.4 ab	0.045*	0.58 n.s	0.013*
Leaf Area (cm^2)	702 ± 10 a	569 ± 19 b	676 ± 23 ab	642 ± 23 ab	< 0.001***	0.38 n.s	0.015*
Tiller Number	16.3 ± 0.9 a	12.8 ± 0.50 b	14.3 ± 0.5 ab	13.2 ± 0.6 b	0.0011**	0.15 n.s	0.073 n.s
Leaf Relative Growth Rate	2.92 ± 0.02a	2.79 ± 0.01c	2.85 ± 0.02b	2.86 ± 0.02b	0.0014**	0.61 n.s	< 0.001***
Root DW (g plant^{-1})	17.8 ± 1.2 a	17.0 ± 0.8 a	17.2 ± 1.1 a	18.5 ± 1.5 a	0.81 n.s	0.68 n.s	0.39 n.s
Root/Shoot ratio	1.6 ± 0.1 a	1.7 ± 0.1 a	1.6 ± 0.1 a	1.8 ± 0.1 a	0.20 n.s	0.69 n.s	0.82 n.s
Maximum Root Length (cm)	55.5 ± 2.5 c	76.0 ± 1.1 b	82.8 ± 1.3 b	98.0 ± 3.4 a	< 0.001***	< 0.001***	0.28 n.s
Total Dry Weight (g plant^{-1})	29.12 ± 1.21 a	26.84 ± 1.00 b	27.61 ± 1.30 ab	29.11 ± 1.65 a	0.77 n.s	0.77 n.s	0.015*
Water Use Efficiency (g l^{-1})	1.69 ± 0.04 ab	1.56 ± 0.05b	1.75 ± 0.03a	1.77 ± 0.02a	0.16 n.s	< 0.001***	0.06 n.s
Phosphorus Use Efficiency (g mg^{-1})	0.57 ± 0.01 c	0.60 ± 0.03 bc	0.69 ± 0.03 ab	0.75 ± 0.03 a	0.09 n.s	< 0.001***	0.69 n.s

Two-way ANOVA results (P-values reported). Treatment effects are: not significant (n.s., $P > 0.05$), $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P < 0.001$(***).

dynamics. Other phytohormones may be involved in regulating leaf expansion. Foliar JA and ACC concentrations both increased under low P (Table 3), with both thought to inhibit monocotyledonous leaf growth (Kim et al., 2015; Tamaki et al., 2015). That effects of low P treatment on vegetative growth depended on AWD irrigation (significant irrigation x phosphorus interactions -Table 7) while JA and ACC concentrations were independent of AWD irrigation (no significant irrigation x phosphorus interactions - Table 3) suggests that changes in both foliar hormone accumulation and sensitivity affect vegetative growth. Moreover, soil drying can induce similar or opposing changes when comparing hormone concentrations in expanded leaves and shoot bases (Todaka et al., 2017), indicating more detailed spatial and temporal analyses are required to understand the regulation of rice vegetative growth with co-occurring P/water deficits.

Stomatal dynamics in response to drying and re-flooding cycles in rice under AWD (Fig. 1b) likely determines plant carbon gain by modulating photosynthesis under mild soil water deficits (Dodd et al., 2015; Song et al., 2018). AWD caused stomatal closure before re-flooding and re-opening after re-flooding that ultimately decreased crop evapotranspiration (Table 2), even if our measurements couldn't distinguish productive (transpiration) and non-productive (evaporation) water use. Plant carbon gain observed in shoot biomass and the decreased crop evapotranspiration leads to an increase in WUE in AWD treatments (Table 2; Table 7). The correlation between Ψ_{leaf} and g_s before re-flooding (Fig. 2a and Table 4) suggests that low Ψ_{leaf} may directly cause stomatal closure of plants exposed to AWD, as previously described (O'Toole and Cruz, 1980; Dingkuhn et al., 1989). However, this response may depend on rice variety, as similar experiments indicate stomatal closure prior to any decrease in Ψ_{leaf} (Siopongco et al., 2008; Parent et al., 2010), suggesting instead that root-sourced signals (such as phytohormones) may cause stomatal closure (Dodd, 2005). Although our measurements could not distinguish whether hormones were root-or shoot-sourced, foliar ABA concentration was negatively correlated with stomatal conductance, while GA1 concentration was positively correlated with stomatal conductance (Fig. 3a and b and Table 5). While previous studies have demonstrated that rice stomatal conductance is negatively correlated with leaf ABA concentration (i.e. Siopongco et al., 2008), its positive correlation with leaf GA1 concentration (Fig. 3b) appears to be a novel response consistent with gibberellins promoting stomatal opening (Dodd, 2003), perhaps related to co-regulation of ABA and GA biosynthesis. While GA1 concentrations were irrigation-dependent (with higher values in CF plants) (Table 3), GA4 concentrations were P-dependent (with higher values in HP plants) (Table 3), implying that these different edaphic stresses may affect the metabolism of GA4 to GA1 (Kobayashi et al., 1993). Paradoxically, the GA insensitive *gid1* rice mutant had increased g_s compared to wild-type plants under drought, suggesting its impaired ability to synthesise ABA overcame its enhanced stomatal sensitivity to ABA (Du et al., 2015). Thus ABA-GA crosstalk, irrespective of whether hormone biosynthesis and/or sensitivity are altered, seems important in regulating rice stomatal conductance.

Perhaps the most exaggerated treatment responses (especially in the LP-AWD treatment) were increases in maximum root length (Table 7). Decreased soil water availability (Shandu et al., 2017; De Bauw et al., 2019a) and low soil P status (Shimizu et al., 2004; De Bauw et al., 2019b) both modify root architecture, thereby enhancing P acquisition and attenuating shoot water deficits. While maximal root length is likely to be of limited significance to plants grown with restricted root systems (as in this study), it may affect resource acquisition of field-grown plants. Puddled rice fields often have a dense layer of soil at depths below the minimum water table height achieved during AWD irrigation (Norton et al., 2017a), which may limit root growth and thus the physiological importance of deeper roots. Instead, increased root length density in the upper soil layers (Yang et al., 2012) may be more important in determining water and nutrient uptake.

Taken together, AWD mitigates the effects of substrate P deficit on shoot growth (biomass, leaf area, tiller number and leaf relative growth rate) thereby enhancing water and phosphorus use efficiencies compared with plants grown under continuous flooding (Table 7). These shoot growth changes appear to be more related to endogenous changes in phytohormone concentrations than AWD increasing P acquisition by increasing substrate P availability or maximal root length (Fig. 6). Although further field trials are needed to understand how soil P dynamics affects crop yields when AWD is applied to soils of contrasting P status, and how AWD regulates P partitioning within the plant, this study suggests that AWD may allow rice growers to decrease P fertiliser rates and irrigation volumes without diminishing vegetative growth.

Contributions

JRAM, performed rice experiment and DGT analyses, statistical analysis, data interpretation and manuscript writing. SAR, performed rice experiments and DGT analyses, data interpretation, and manuscript writing. MJM, performed DGT analyses and its corresponding data analysis. AA, carried out the hormonal analysis and its corresponding data analysis. HZ, coordinated the study and was involved in data interpretation. ICD, coordinated the study and was involved in data interpretation and manuscript writing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

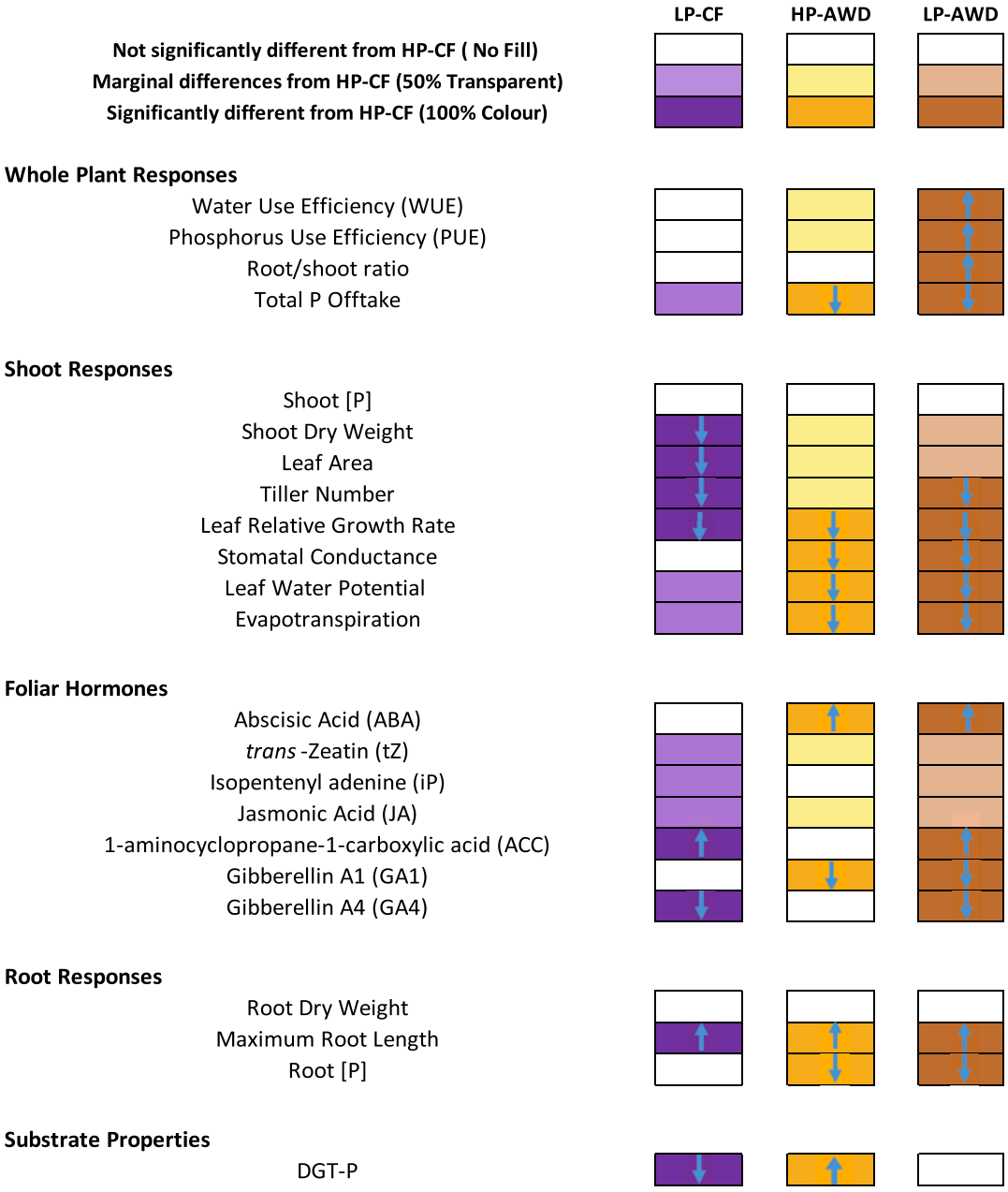

Fig. 6. Schematic diagram of changes in substrate properties and plant responses to AWD irrigation and phosphorus. Colours are violet for LP-CF, orange for HP-AWD and brown for LP-AWD, with white (not filled) for no significant difference, 50% transparent for marginal differences and opaque (100% colour) for significant ($P < 0.05$) differences compared to the HP-CF treatment. Upwards and downwards arrows within each box indicate significant increases or decreases relative to the HP-CF treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.06.017.

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