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Effects of Phosphorus Deficiency on Leaf Surface Morphology: Absorption and Translocation of Foliar-Applied Phosphorus in Four Barley Cultivars

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ABSTRACT

Plant nutrient deficiencies can modify leaf surface properties and may affect the absorption of foliar fertilisers. This study examined how plant P-deficiency modified the adaxial leaf surface morphology of four barley cultivars and whether these modifications could be linked to quantitative differences in foliar-applied P absorption. Four Australian barley cultivars were grown hydroponically under P-sufficient and P-deficient conditions. A ^{32}P radiolabelled foliar phosphate solution was applied to adaxial leaf surfaces for 2 or 7 days to investigate absorption and translocation. All cultivars showed different responses to P-deficiency (stomatal density, trichome density, thickness of the epidermal cell wall and cuticle). However, no clear trends were observed among the cultivars in their responses to P deficiency. Cultivars absorbed foliar-applied P regardless of plant P status. Remobilisation occurred from the treated leaf to untreated shoots in all but one cultivar. While P-deficient plants absorbed and accumulated significantly less foliar-applied P after 7 days, this was not linked to measured changes in stomatal or trichome density or the thickness of the epidermal cell wall and cuticle. Autoradiographs revealed that ^{32}P accumulation was limited to newly emerging leaves in P-deficient plants, while P-sufficient plants also remobilized and accumulated ^{32}P into older leaves and tillers. Relatively high P absorption (> 65% of foliar-applied P) in both P-sufficient and P-deficient plants suggests that foliar-applied P may be a useful fertiliser top-up strategy for barley. Due to the lower absorption in P-deficient barley, foliar applications should be made before severe P-deficiency symptoms are apparent to improve absorption.

1 | Introduction

Phosphorus (P) is an essential macronutrient for plant growth and function (de Bang et al. 2021). Since biogeochemical processes in the soil limit root P uptake, excessive, compensatory soil P applications are common, increasing the risk for subsequent nutrient run-off or leaching into groundwater, which at the global scale can threaten the stability of regulating planetary boundaries (Zou et al. 2022; Rockström et al. 2023). Despite high fertiliser inputs, approximately 30% of the world's

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croplands also suffer from soil P deficiency (MacDonald et al. 2011). As agriculture accounts for approximately 90% of mineral P demand, P use efficiency (PUE) needs to increase by as much as 80% in some regions to address these complex challenges (Koppelaar and Weikard 2013; UN Department of Economics and Social Affairs 2015; Zou et al. 2022). While P is commonly applied as a soil fertiliser at sowing, the PUE of this strategy is often low due to biogeochemical soil processes, especially in highly fixing soils such as those with high pH and calcium content or low pH and rich in iron and aluminium (Roberts and Johnston 2015) or due to microbial immobilisation when the C:P ratio of organic residues is > 300:1 (Richardson and Simpson 2011), resulting in reduced P available for plant uptake. The high inputs of P fertilisers to soils contribute 38% of total P anthropogenic inputs into the earth system (Kopittke et al. 2021).

Targeted 'top-up' sprayings during the growing season using foliar-applied P fertilisation may be an attractive strategy, as this could permit a lower amount of P to be applied to the soil at sowing, meeting plant demand according to plant growth stage and seasonal conditions (Noack et al. 2010). However, the literature has reported variable results following foliar-applied P applications. Studies have shown high recovery of foliar-applied P in wheat, although only a few foliar formulations translated into increased plant biomass or grain yields (McBeath et al. 2011, 2020; Peirce et al. 2014, 2016). Multiple foliar sprays in combination with seed dressings were more efficient than only soil-based fertilisation in spring wheat, suggesting that several applications may be required to improve grain yield (Talboys et al. 2020). Similarly, foliar P applications in juvenile maize increased plant P concentrations for 10 days following spraying, but there were no effects on yield at harvest (Görlach, Henningsen, et al. 2021). P absorption was also reported to occur from foliar-deposited desert dust in P-deficient chickpea and wheat, where leaf surface acidification processes were linked to P solubilisation and subsequent increases in crop biomass (Gross et al. 2021; Duarte et al. 2023).

Designing effective foliar fertiliser formulations requires an understanding of how foliar-applied solutes penetrate leaf surfaces, which may occur through the cuticle, trichomes stomata, and/or leaf veins (and associated fibre cells and bundle sheath extensions) (Fernández et al. 2021; Schlegel and Schönherr 2002). The cuticle is a protective biocomposite layer covering the surface of aerial plant parts and may be considered a modified cell wall or membrane, containing a range of hydrophobic (lipids such as waxes, cutin, cutan) and hydrophilic functional groups and compounds (e.g., polysaccharides) that are present in a high variation of assorted abundances and nanoscale complex arrangements (Niklas et al. 2017; Fernández et al. 2021). The highly heterogeneous physical and chemical structure of the cuticle complicates understanding the exact mechanisms behind water and solute absorption. Water transport was reported to be linked to a dynamic 'polar continuum' forming across areas of higher polysaccharide abundances (either in a gradient increasing towards the leaf interior or through random arrangements), cutin abundances in the cuticle exterior, and the level of cuticle water sorption and swelling (in which cellulose plays an important role) (Fernández et al. 2017; Kamtsikakis et al. 2021; Tredenick and Farquhar 2021; Heredia et al. 2024). The theorised existence of 'aqueous pores' facilitating water or solute absorption across the cuticle has been challenged due to the current lack of evidence; solute diffusion may instead be associated with the presence of polysaccharides in the cuticle and aided by the swelling of the cuticle in the presence of water or high relative humidity (RH) (Fernández et al. 2021; Guzmán-Delgado et al. 2021; Heredia et al. 2024).

Trichomes are single-celled or multicellular epidermal structures present on aerial plant parts and have been shown to be the main pathway for the absorption of ionic and fluorescent tracers in beech leaves due to the reported lack of a cuticle (or potentially a cuticle containing lower abundances of lipids) and the presence of an external pectin-rich cell wall layer (Rubinstein et al. 2020). The first studies visualizing foliar-applied P absorption pathways showed fibre cells, and trichomes were dominant pathways for absorption in spring barley leaves, with some contribution from stomatal and cuticular penetration (Arsic et al. 2020, 2022). Fibre cells are sub-epidermal sclerenchyma cells occurring in the periphery to vascular bundles at the nexus of the epidermis and the bundle sheath extension (Arsic et al. 2020). Trichomes and cells overlying leaf veins were important for foliar P absorption in tomato, as well as for zinc (Zn), iron (Fe), manganese (Mn) and calcium (Ca) absorption in multiple crops (Du et al. 2015; Li et al. 2018, 2019, 2021; Henningsen, Bahamonde, et al. 2023).

Stomatal solute penetration may occur via diffusion through a continuous liquid film alongside stomatal walls, or through the anticlinal cell walls of the guard cells, due to their reported higher porosities and wettability, although this has not yet been validated experimentally (Eichert et al. 2008). Stomata can contribute to foliar solute absorption for sodium chloride (NaCl) salts and nitrogenous compounds (Eichert and Goldbach 2008; Burkhardt et al. 2012). Stomatal penetration of water and solutes is contentious: it is possible that solute transport via diffusion may be independent of the solvent; nanoparticles and solutes may penetrate the stomatal pore via diffusion along guard cell surfaces, even if water penetration may be largely occurring via water vapour diffusion (Fernández et al. 2021; Guzmán-Delgado et al. 2021). Solutes such as nutrient ions may then be absorbed via the stomata through diffusion and mass flow, at least if surfactants are added.

As latent P-deficiency is common in agricultural soils, plant P-deficiency effects on leaf surface structure could also affect foliar-applied nutrient absorption, with variable results shown among studied crop species (Carstensen et al. 2019). For example, severely P-deficient wheat plants did not absorb any foliar-applied P within 48 h due to reduced stomatal and trichome densities, total leaf area, and cuticle thickness (Fernández et al. 2014). In maize, P-deficiency altered the chemical properties of the leaf surface (increased proportions of cutin and waxes), with no effect on stomatal densities or leaf wettability, although foliar-applied P increased tissue P concentrations and net photosynthesis rates in all four varieties tested (Henningsen, Bahamonde, et al. 2023). Phosphorus-deficient maize plants absorbed foliar-applied P within 6 h into metabolically active pools in the cytosol, as rapid remobilization to younger shoots occurred, and there was no evidence of P storage in the leaf apoplast (Görlach, Henningsen, et al. 2021; Görlach, Sagervanshi, et al. 2021).

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Conversely, P-deficiency in barley was reported to have negligible effects on stomatal or trichome densities; however, the cuticle and epidermal cell wall were modified (increased thickness and lower abundance of polysaccharides) (Arsic et al. 2020, 2022). This impacted the absorption of foliar-applied P: foliar-applied P was unable to restore photosynthetic function within 6 h when applied to leaves either at tillering or flag leaf emergence (Arsic et al. 2022). The length of time allowed for foliar absorption is likely to play a major role in absorption, as foliar-P applications successfully restored photosynthetic functionality after 24 h in young P-deficient spring barley (Arsic et al. 2020). However, several gaps were identified in these studies. First, it had not been determined whether plant P deficiency quantitatively affected the absorption of foliar-applied P due to high background P concentrations in plant tissue. Second, the effect of a thicker cuticle and epidermal cell wall in P-deficient plants on foliar-P absorption was also unclear. Many studies have shown that cuticle thickness was not the determining factor for cuticular penetration, and cell wall thickness was but one factor affecting mass transport and diffusivity (e.g., substance size constraints, transport pathway length, membrane structure and composition) (Norris 1974; Schonherr and Riederer 1989; Baur et al. 1997; Buchholz et al. 1998; Schreiber 2009). However, the thickness of the cuticle and epidermal cell wall has been linked to the reduced absorption of Zn, Mn and Fe in sunflower and tomato leaves, possibly through increased path length or tortuosity (Li et al. 2017). Mn deficiency in barley also led to a thinner cuticle and epidermal cell wall containing lower abundances of waxes (Arsic et al. 2022). Therefore, there was interest in determining whether the thickness of the cuticle and epidermal cell wall could be linked to quantitative differences in foliar-applied P absorption in barley. Thirdly, as published studies in barley examined only one cultivar, it remained unclear whether the reported modifications of leaf surface properties and foliar-P absorption would be observed in multiple commercially available cultivars.

Therefore, there is a need to identify whether plant P deficiency can be linked to specific changes to leaf surface morphology in barley and whether these changes could be linked to quantitative differences in foliar-applied P absorption. In this paper, foliar absorption refers to processes where foliar-applied solutes move across the leaf surface into plant tissues, where this is the first rate-limiting step for the utilisation of these applied solutes by plants. The term 'absorption' is used broadly to refer to the presence of foliar-applied solutes in the whole plant, as solutes must have been absorbed across the leaf surface to be detected in any plant part. Absorbed solutes may then be translocated and accumulate in various cells, tissues, and plant parts. The translocation and accumulation of absorbed foliar-applied P is also important to understand, as these processes provide information about the availability of the absorbed nutrients to be utilised in certain metabolic processes (e.g., accumulation in shoots may indicate availability of absorbed nutrients to restore photosynthetic machinery limited by the low availability of phosphate ions in P deficient plants (Carstensen et al. 2018; Arsic et al. 2020)).

The aim of this study was to compare the effects of plant P deficiency on the adaxial leaf surface morphology of four Australian barley cultivars, and whether such modifications can be linked to quantitative changes in the absorption of foliar-applied P in whole plants. Methods included assessing leaf surface morphology (trichome and stomata densities, epidermal and cell wall thickness) and the absorption, translocation and accumulation of foliar-applied ^{32}P via liquid scintillation counting and autoradiography.

2 | Materials and Methods

2.1 | Plant Growth

Four commercial Australian barley cultivars (Flagship, Gairdner, Macquarie and Westminster) were selected to cover a range of stomatal and trichome densities (Hasanuzzaman et al. 2017). The four varieties were grown under P-sufficient and P-deficient conditions in a hydroponic rooting medium. Barley seeds were germinated in Sorbix vermiculite for 5 days at The University of South Australia (Mawson Lakes, Australia) in a controlled temperature greenhouse, with average daytime temperatures of 23°C ± 5°C and 15°C ± 4°C at night. Uniform seedlings were selected and transferred to aerated and light impermeable 0.75 L hydroponic units. Each unit had a lid with 3 holes (one seedling was held per hole using plastic hydroponic tubing), containing three randomised different cultivars grown in a chelate-buffered solution prepared in 18.2 MΩ/cm ultrapure water (Milli-Q Plus; Millipore). Two types of nutrient solutions were prepared to produce either P-sufficient or deficient conditions in the hydroponic medium. P-sufficient plants were grown with the following nutrients: 200 μM KH_2PO_4, 200 μM K_2SO_4, 300 μM $MgSO_4{\cdot}7H_2O$, 100 μM NaCl, 300 μM $Mg(NO_3)_2{\cdot}6H_2O$, 900 μM $Ca(NO_3)_2{\cdot}4H_2O$, 600 μM KNO_3, 50 μM Fe(III)-EDTA-Na, 1.0 μM $MnCl_2{\cdot}4H_2O$, 0.8 μM $Na_2MoO_4{\cdot}2H_2O$, 0.7 μM $ZnCl_2$, 0.8 μM $CuSO_4{\cdot}5H_2O$, 2.0 μM H_2BO_3. P-deficient plants were supplied with low P concentrations, where 200 μM KH_2PO_4 was replaced with 9 μM KH_2PO_4 and 200 μM KCl (all reagents obtained from Sigma Aldrich) (Arsic et al. 2020). Hydroponic solutions were changed every week until week 4 and then twice per week. The pH was adjusted to 6.0 ± 0.5 with 1 M HCl or 1 M NaOH every second day. Four hydroponic units were prepared for each hydroponic plant P status (P+ or P−), to ensure three independent biological replicates of each cultivar per P status. Hydroponic units were arranged in a fully randomised design, and positions were randomised every week.

2.2 | Microscopy

Leaf samples (1 × 1 cm) were collected at 30 days after sowing (DAS) from the middle portion of the youngest fully expanded leaf (YFEL) of untreated P-sufficient and P-deficient plants (corresponding to the leaf stage when ^{32}P was applied). Samples were fixed in Karnovsky's fixative (EMS, Hatfield), washed in 0.1 M sodium cacodylate buffer (Sigma Aldrich; pH 7.3) and deionised water, and dehydrated in a graded acetone dehydration series (Arsic et al. 2020). Samples for scanning electron microscopy (SEM) analysis were sputter coated with a mixture of platinum and gold and then examined under the microscope (Zeiss Merlin FEG SEM). Samples were used to determine stomatal and trichome densities. For histological analysis, smaller YFEL sections (5 × 10 mm) underwent the same fixation and

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washing process followed by an ethanol dehydration series before infiltration and embedding in LR (London Resin) White (EMS, Hatfield). 2.5 μm thin cross sections were obtained using a Leica EM UC7 ultramicrotome coupled to a Nikon Eclipse Ni microscope and DS-Fi1c camera. Sections were stained using Toluidine Blue O (TBO) for 30 s.

2.3 | Foliar ^{32}P Application

At 30 DAS, hydroponic units were moved to a growth chamber inside a certified radiation laboratory, set to a 12 h day/night cycle at 25°C/20°C and RH at 75%. Foliar P was applied in two batches according to the two lengths of droplet application time (48 h and 7 days) due to limited space in the growth chamber: one subset of 30 DAS plants was set up in the growth chamber, and foliar P droplets were applied and left for 48 h. These plants were then harvested and processed. A new subset of 30 DAS plants was moved into the growth cabinet from the glasshouse, where foliar P droplets were applied and left for 7 days before the plants were removed and processed.

The foliar-applied P solution consisted of 0.15 mol L^{-1} (2% w/v) diammonium phosphate (DAP) ($(NH_4)_2HPO_4$; Sigma Aldrich) and 0.05% (v/v) Tween-20 (adjusted to pH 6). This solution was labelled with carrier-free orthophosphate ^{32}P (PerkinElmer), resulting in a spike rate of 30 kBq $plant^{-1}$ at the time of application. Ten 5 μL droplets were applied to the upper (adaxial) side of the middle portion of the YFEL using a micro-dosing pipette. The rate of foliar-applied P was 230 μg P $plant^{-1}$. Droplet size was consistent upon application, and treated leaves were marked for identification. As mentioned above, droplets were applied and left for either 48 h or 7 days. There were four biological replicates for each treatment (e.g., per combination of plant P status, cultivar and application time), where three plants were used for tissue ^{32}P quantification, and one plant was used for autoradiography (see details in the section below). Spike blank samples (i.e., 5 μL droplets of ^{32}P spiked DAP and 0.05% (v/v) Tween-20) solution were also prepared at both application periods (48 h and 7 days, $n = 6$) to measure the exact specific activity of the applied solutions.

2.4 | Tissue ^{32}P Leaf Sampling and Analysis

After 48 h, treated leaves were blotted using tissue paper to prevent cross-contamination of samples whilst removing plants from the growth chamber, as droplets were still wet (Figure S1). The 7 days treated leaves did not require blotting. Following both exposure periods, whole plants were harvested and divided into the treated leaf, non-treated leaves, tillers and roots. The treated leaves were washed sequentially using three rinse solutions: first in 2% (v/v) HNO_3 (Sigma Aldrich), then 3% (v/v) ethanol and finally deionised water, where all solutions contained 0.05% (v/v) Tween-20 (Arsic et al. 2022). Rinse solutions were combined and analysed for ^{32}P via liquid scintillation counting. Plant parts were oven-dried for at least 48 h at 70°C, and all tissue weights were recorded. Treated leaves and roots were cut into small pieces and digested. Untreated shoot material was initially homogenised via grinding before sub-samples were weighed and digested. Plant material was cold digested overnight in 5 mL 69% (w/w) HNO_3 (Sigma Aldrich), after which 1 mL 30% (w/w) H_2O_2 (Sigma Aldrich) was added, and the material was digested at 100°C using a hot block until near dryness (< 1 mL). Samples were diluted to a total volume of 20 mL with 5% (v/v) HNO_3 and passed through 0.45 μm syringe filters (0.45 μm MCE syringe filter, MF-Millipore). A 3 mL aliquot of each digest solution and 16 mL of Ultima Gold AB (PerkinElmer) scintillant were added to glass scintillation vials. The ^{32}P activity in each sample was measured using a Tri-Carb Liquid Scintillation Counter (Packard). All counts were blank and decay corrected to a single time point. The amount of foliar-applied P absorbed and translocated was calculated as a percentage of the total foliar P applied at each time point (Peirce et al. 2019). Plant material and spike blanks were also analysed for total P using an Agilent 8800 Triple Quad ICP-MS in O_2 mode. All samples were blank-corrected, and NIST tomato leaves were used as a plant material reference standard (SRM 1573a, National Institute of Standards and Technology (NIST), USA). The standard reference material recovery for P was 103% (2245 ± 297 ug g^{-1} P, $n = 17$).

2.5 | ^{32}P Autoradiography

Autoradiography was performed on dried whole plants (one plant per treatment) to visualize ^{32}P accumulation and translocation. The treated leaf was blotted using tissue paper and rinsed using the same solutions outlined above before whole plants were mounted onto cardboard sheets, fixed with double-sided scotch tape, and dried in an oven at 70°C for at least 48 h. The adaxial side of the treated leaf was visible in all samples. Mounted samples were then enveloped inside an 80 μm thick, clear plastic sheet to prevent cross-contamination and sample loss during transport. Samples were transported in a double-walled container (Perspex) to the Australian Nuclear Science and Technology Organisation (ANSTO) Biosciences facility at Lucas Heights, Australia. Whole, dried plants were exposed to BAS-SR 2040 (Fuji Film) imaging phosphor (IP) plates inside a standard imaging cassette. Samples were removed from their envelope, and a 9 μm Mylar film was placed between the sample and the IP plate to prevent cross-contamination. The plate was exposed for 1 h, and then the resulting phosphorescence was imaged using a FLA7000 scanner (GE). Two lengths of foliar application times were analysed: 48 h and 7 days. Autoradiographic images were processed using ImageJ Fiji. To correct for slight differences between plates and exposure times, a set of two standards was created covering a range of 3760 to 0.376 Bq cm^{-2} by serial dilution onto aluminium backed silica gel TLC plates. These standards were included in each sample exposure. The quantity of radioactivity for these standards was decay corrected for each image, and the equivalent image thresholds were set accordingly. This semi-quantitative approach allows for direct comparisons of radioactivity to be made across the various images between parts of the plants that are anatomically similar.

2.6 | Statistical Analysis

Data analysis was conducted using GraphPad Prism version 8.3.1 for Windows. Analysis of variance (ANOVA) was used to determine significant differences between treatments, where post hoc Tukey's or Šídák's multiple comparisons tests were

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used to compare the treatment groups for significant differences. Data transformations were applied when necessary to ensure datasets met the assumptions of normality and homoscedasticity. Significant differences were set to $p < 0.05$ (α).

3 | Results

3.1 | Effect of Plant P Status and Cultivar on Biomass

Plant P status had a strong effect on plant biomass production (Table 1). P-deficiency reduced the dry shoot biomass by 70%–75% and the dry root biomass by 50%–70% across all cultivars (Table 1). P-deficient plants had fewer tillers and showed signs of anthocyanosis (see Figure 7). The P-deficient plants also had at least double the root-to-shoot ratio compared to P-sufficient plants (Table 1). The P-sufficient Flagship cultivar had significantly greater shoot biomass than the other P-sufficient cultivars, while all P-deficient cultivars had similar shoot biomasses (Table 1). The P-deficient Flagship and Westminster cultivars had significantly lower root biomass compared to their P-sufficient controls (Table 1). Shoot P concentrations were significantly higher in P-sufficient compared to P-deficient cultivars, which fell below the widely accepted tissue P threshold concentration for P-sufficiency (2000 $\mu g\,g^{-1}$ dry matter [DM]) (Table 1) (Reuter and Robinson 1997). P-deficient plants had fewer tillers and leaves that were dark green and much smaller compared to P-sufficient plants (see Figures S2 and 7).

3.2 | Effect of Plant P Status and Cultivar on Adaxial Leaf Surface Properties

The adaxial leaf surface had files of epidermal cells running parallel to the leaf margin, typical of grass species (Ellis 1979) (Figure 1). Undulating ridges and valleys followed leaf venation, with trichomes typically found on top of vein ridges and stomata on descending sides of the interveinal valleys (Figure 1). While trichomes have previously only been observed on top of vein ridges in barley (Arsic et al. 2020), P-deficient Flagship and P-sufficient Macquarie and Westminster also had smaller trichomes occurring in the interveinal regions (Figure 1b,e,g). These interveinal trichomes were also observed in very low densities in P-sufficient Flagship (4%) and Gairdner (2%) (data not shown), but not in other P-deficient cultivars (Figure 1).

Trichome densities differed significantly by cultivar ($p = 0.0002$) and plant P status ($p < 0.0001$), and there was also a significant interaction between factors ($p < 0.0001$) (Figure 2a). Under P-sufficient conditions, Flagship had the lowest trichome densities ($25 \pm 3\,mm^{-2}$) while Macquarie had the highest densities ($67 \pm 2\,mm^{-2}$), likely due to the presence of very high densities of small interveinal trichomes (42% of total trichomes) (Figure 2a). Under P-deficient conditions, trichome densities decreased in Macquarie ($24 \pm 8\,mm^{-2}$) while an increase was observed in Flagship ($41 \pm 5\,mm^{-2}$), also likely due to the presence of interveinal trichomes (20% of total trichomes) (Figure 2a). Stomatal densities also differed significantly due to cultivar ($p = 0.0061$) and plant P status ($p = 0.002$), but there was no significant interaction between factors (Figure 2b). Between P-sufficient and P-deficient cultivars, only Macquarie showed a significant decrease in stomatal densities (103 ± 2 to $80 \pm 13\,mm^{-2}$) (Figure 2b). While there were no significant differences among P-sufficient cultivars, among P-deficient cultivars, Gairdner had lower stomatal densities ($71 \pm 4\,mm^{-2}$) compared to Flagship ($94 \pm 4\,mm^{-2}$) (Figure 2b).

Staining of embedded leaf cross-sections with TBO was used to determine differences in epidermal cuticle and cell wall thickness and to illustrate general leaf anatomy (Figure 3). TBO is a polychromatic dye, staining lignified walls and sclerenchyma blue-green, collenchyma and parenchyma reddish purple, and sieve tubes and companion cells red (O'Brien et al. 1964). Fibre cells and trichomes appeared to be stained blue in color compared to pavement epidermal cells (Figure 3). Small interveinal trichomes were observed in P-deficient Flagship (Figure 3b) and P-sufficient Macquarie (Figure 3e), also seen in SEM micrographs (Figure 1b,e). They did not appear to be adjacent to sub-epidermal fibre cells, unlike the trichomes occurring on top of vein ridges (e.g., Figure 3a), where the small trichome in the Macquarie leaf cross-section appeared to be located above a possible sub-stomatal cavity (Figure 3e).

Epidermal cuticle and cell wall thickness showed significant plant P status ($p < 0.0001$) and interaction effects ($p = 0.0017$) (Figure 4).

TABLE 1 | Total dry biomass production and shoot tissue P concentration in control P-sufficient and P-deficient barley cultivars. Data represent the mean values ± SE ($n = 6$ for biomass data, $n = 3$ for total P data). Means with the same letter are not significantly different ($p < 0.05$).

P-status	Cultivar	Shoot biomass (g)	Root biomass (g)	Root/shoot	Shoot [P] ($\mu g\,g^{-1}$ DM)
P+	Flagship	2.3 ± 0.2^{a}	0.24 ± 0.03^{a}	0.11 ± 0.01^{b}	2990 ± 257^{a}
	Gairdner	1.5 ± 0.1^{b}	0.17 ± 0.03^{abc}	0.11 ± 0.01^{b}	3078 ± 130^{a}
	Macquarie	1.7 ± 0.1^{b}	0.19 ± 0.02^{ab}	0.12 ± 0.02^{b}	3685 ± 303^{a}
	Westminster	1.6 ± 0.1^{b}	0.24 ± 0.02^{a}	0.15 ± 0.01^{b}	3029 ± 155^{a}
P−	Flagship	0.49 ± 0.05^{c}	0.13 ± 0.01^{bc}	0.29 ± 0.03^{a}	1046 ± 236^{b}
	Gairdner	0.37 ± 0.03^{c}	0.12 ± 0.02^{bc}	0.32 ± 0.04^{a}	1052 ± 282^{b}
	Macquarie	0.36 ± 0.03^{c}	0.11 ± 0.01^{cd}	0.31 ± 0.02^{a}	910 ± 145^{b}
	Westminster	0.35 ± 0.04^{c}	0.13 ± 0.02^{bc}	0.37 ± 0.02^{a}	619 ± 35^{b}

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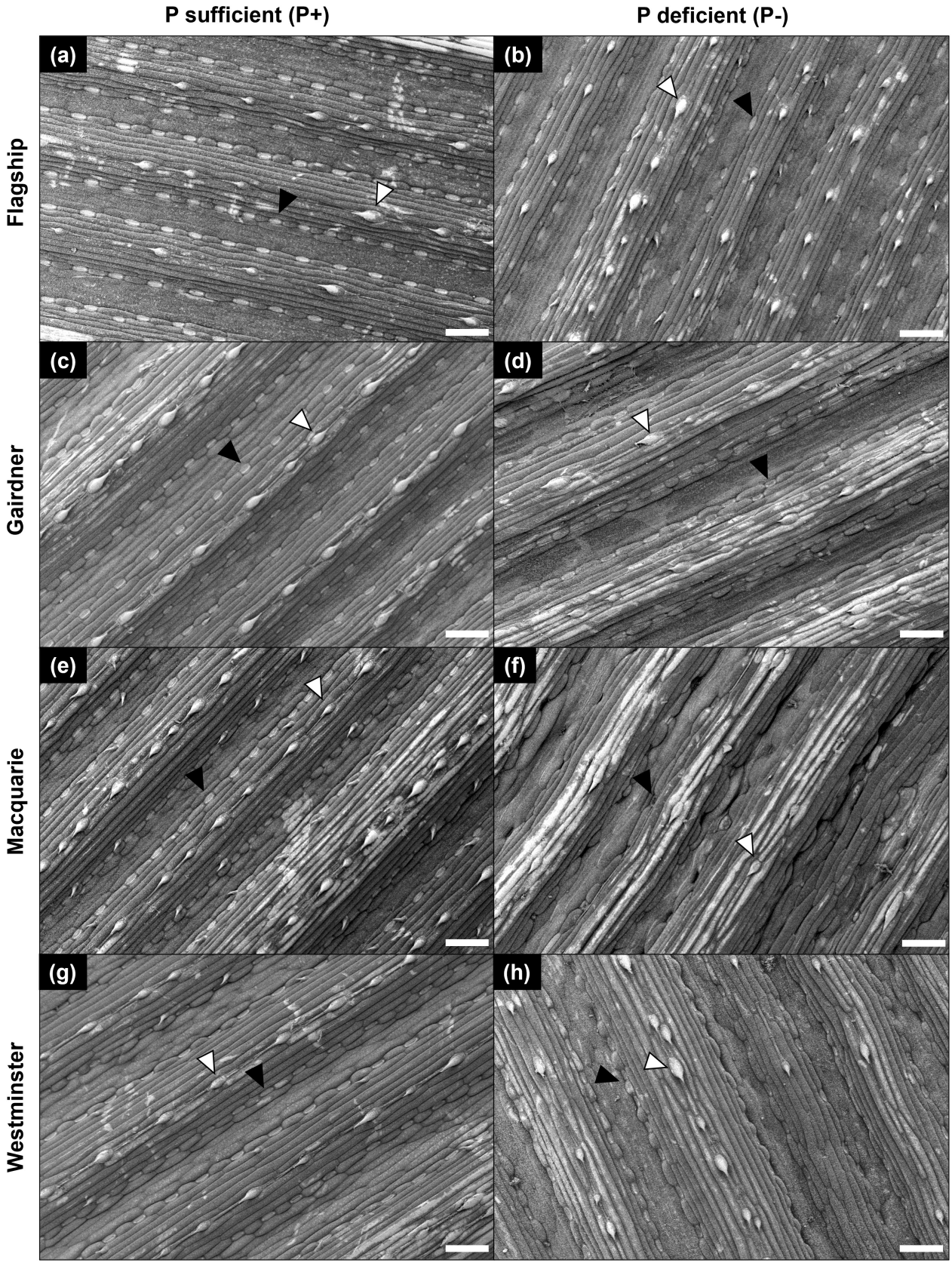


FIGURE 1 | Representative SEM micrographs of adaxial barley leaf surfaces. Flagship (a) P-sufficient and (b) P-deficient; Gairdner (c) P-sufficient and (d) P-deficient; Macquarie (e) P-sufficient and (f) P-deficient; Westminster (g) P-sufficient and (h) P-deficient YFEL. Black arrows indicate stomata, while white arrows indicate trichomes. Scale bars = 100 μm.

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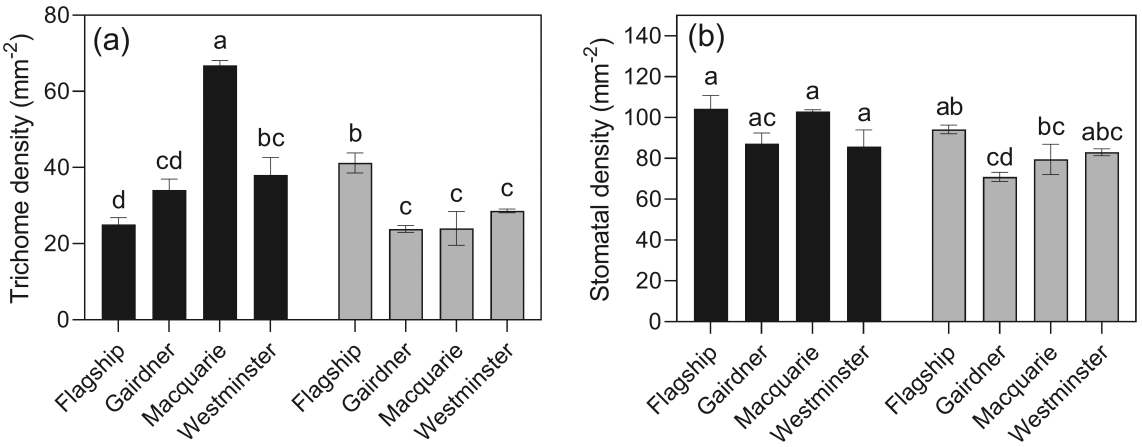


FIGURE 2 | Adaxial densities of (a) stomata and (b) trichomes in P-sufficient (black bars) and P-deficient (grey bars) barley cultivars per mm^2. Error bars show SE ($n = 3$). Different letters indicate significant mean differences across all treatments ($p < 0.05$).

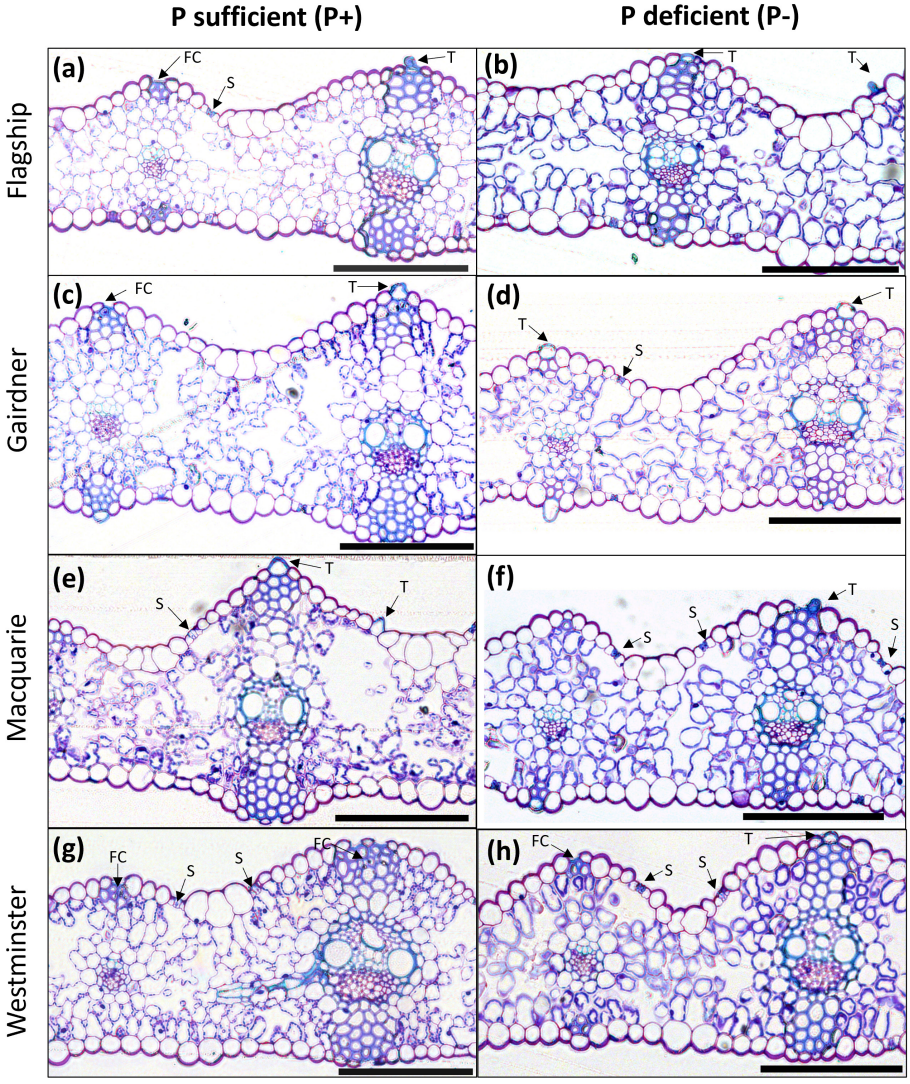


FIGURE 3 | Representative leaf cross-sections stained with Toluidine Blue O for the barley varieties Flagship (a) P-sufficient and (b) P-deficient; Gairdner (c) P-sufficient and (d) P-deficient; Macquarie (e) P-sufficient and (f) P-deficient; Westminster (g) P-sufficient and (h) P-deficient YFEL. FC = fibre cell, S = stomate, T = trichome. Scale bars = 100 μm.

Among P-sufficient cultivars, Flagship had a thicker cuticle and epidermal cell wall than Macquarie, while among P-deficient cultivars Macquarie and Westminster had significantly thicker cuticle and epidermal cell walls compared to Gairdner (Figure 4). When comparing the difference between P-sufficient and P-deficient cultivars, P-deficient Macquarie and Westminster plants had

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significantly thicker cuticle and epidermal cell walls compared to their respective P-sufficient plants (Figure 4).

3.3 | Foliar-Applied P Absorption

Between 31%–55% of the foliar P applied had been absorbed by all cultivars after 48 h, and between 65%–88% was absorbed after 7 days (Table 2). While P-deficient cultivars had lower mean ^{32}P absorption than P-sufficient cultivars, there was high variability in the treatments, which resulted in large standard errors (Figure 5a). When combining the ^{32}P measured in plants and in rinse solutions, total ^{32}P recovery was very high after 7 days (up to 95% of applied ^{32}P) (Table S1). It was not possible to make mass balance comparisons for the 48 h treatments, as the necessary blotting of the wet droplets prior to plant removal from the growth chamber (to prevent cross-contamination of liquid droplets on other plant parts) meant that some applied ^{32}P was removed on the blotting tissue paper.

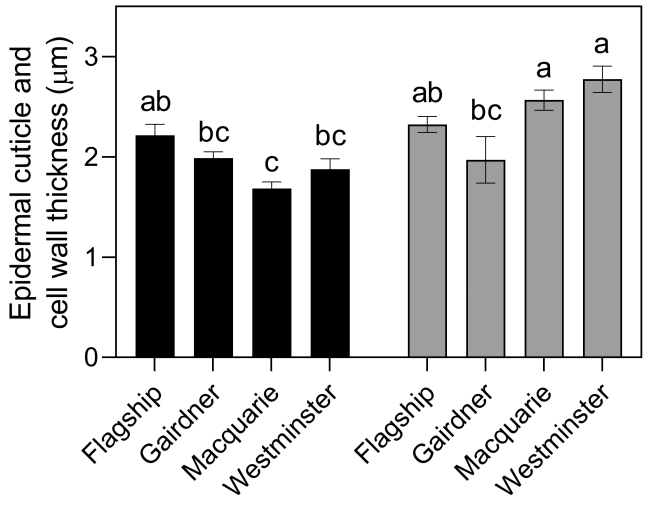


FIGURE 4 | Cuticle and epidermal cell wall thickness for barley cultivar adaxial YFEL surfaces in P-sufficient (black bars) and P-deficient (grey bars) plants. Error bars show SE ($n = 3$). Different letters indicate significant mean differences across all treatments ($p < 0.05$).

After 48 h, there were no significant differences in foliar-applied P absorption due to plant P status or cultivar (Figure 5a). After 7 days, P-sufficient plants had absorbed significantly more foliar-applied P than P-deficient plants (plant P status, $p = 0.0365$) (Figure 5b). However, a Tukey's post hoc multiple comparisons test did not attribute this to a specific grouping of cultivars (Figure 5b).

To summarise the differences between cultivars due to P-status, percentage differences were calculated for leaf surface properties and foliar-applied P absorption (P-deficient/P-sufficient) (Table 2). The results showed that each cultivar had different modifications of adaxial leaf surface properties when comparing P-sufficient and P-deficient plants. The Flagship cultivar had a significant increase in trichome density (165%), but no significant difference in stomatal density or cuticle and epidermal cell wall thickness. While Gairdner had no significant differences in any leaf surface properties, P-deficient Macquarie showed significantly lower trichome and stomatal densities as well as a significantly thicker cuticle and epidermal cell wall compared to P-sufficient plants. P-deficient Westminster had a significantly thicker cuticle and epidermal

TABLE 2 | Percentage change between P-deficient and P-sufficient plants—adaxial leaf surface properties and foliar-applied ^{32}P absorption (^{32}P concentrations in whole plants divided by foliar-applied ^{32}P concentrations). Significant differences between P-sufficient and P-deficient means are noted by an asterisk.

Cultivar	Trichomes (%)	Stomata (%)	Epidermal thickness (%)	48 h ^{32}P absorption (%)	7 days ^{32}P absorption (%)
Flagship	165*	91^{ns}	105^{ns}	84	102
Gairdner	70^{ns}	81^{ns}	99^{ns}	60	88
Macquarie	36*	77*	152*	68	77
Westminster	75^{ns}	97^{ns}	148*	64	89

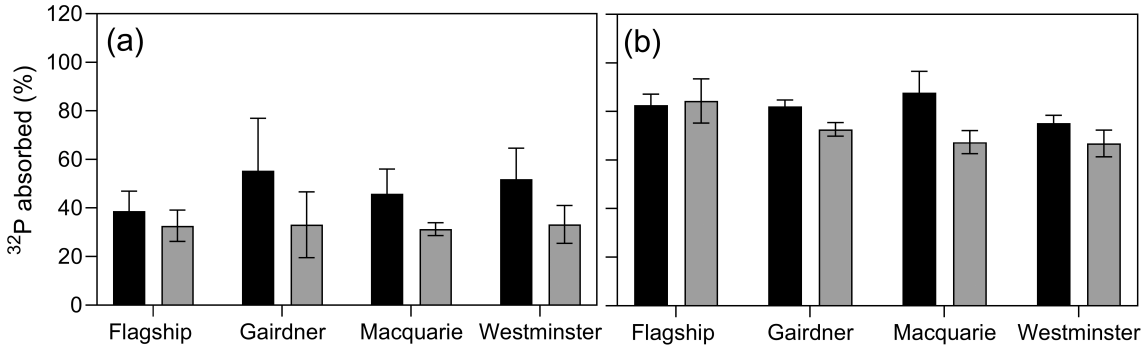


FIGURE 5 | Foliar-applied ^{32}P absorbed in all plant parts after (a) 48 h and (b) 7 days in P-sufficient (black bars) and P-deficient (grey bars) plants. Error bars show SE ($n = 3$).

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cell wall but no significant differences in trichome or stomatal densities. There were no significant differences in foliar-applied P absorption after 48 h due to the high variability in measurements. There was significantly lower absorption of foliar-applied P after 7 days in P-deficient cultivars, although this could not be statistically attributed to specific cultivars (Table 2). Even though the statistical significance of the results for ^{32}P absorption is limited, P uptake was consistently lower (with only one exception) for P-deficient plants (Table 2). The error in the determination of ^{32}P uptake was most likely the result of the propagation of error caused by the multiple analyses required to calculate this parameter (i.e., the % of ^{32}P uptake is the result of three independent sample weighing, digestion and beta counting for each sample, a total of 27 procedures that can introduce variation for three replicates).

3.4 | Translocation and Accumulation of Foliar-Applied ^{32}P

Foliar-applied P was translocated to all parts of the plant after 48 h (Figure 6a). Most of the absorbed foliar-applied P accumulated in the treated leaf (26–45%), with small amounts of foliar-applied P accumulating in the untreated shoots (4%–10%) and roots (0.8%–1.5%) (Figure 6a). There were no significant differences due to plant P status when comparing the treated leaf, untreated shoots, or roots. After 7 days, most (> 65%) of the applied foliar P had been absorbed from the foliar-applied solution (Figure 6b). There were no significant differences between the amount of accumulated foliar-applied P in the treated leaf (32%–50%) or the untreated shoots (23%–46%), except for P-deficient Macquarie (Figure 6a,b). P-deficient Macquarie had significantly less applied foliar-P that accumulated in untreated shoots compared to other cultivars (14.3% ± 12%) (Figure 6b). Foliar-applied P had also been translocated to the roots of all cultivars (3%–7%) (Figure 6b).

Autoradiography of dried whole plants showed subtle differences in the translocation and accumulation of foliar-applied ^{32}P after 48 h between cultivars and due to plant P status (Figure S2). ^{32}P was primarily translocated upwards and accumulated towards the leaf tip, thus following the transpiration stream. P-sufficient Macquarie also showed translocation downwards and accumulation in the base of the treated leaf and down the tiller (Figure S2j). P-deficient Gairdner, Macquarie and Westminster appeared to show higher translocation and accumulation of ^{32}P to the crowns (where the stems meet the roots) (Figure S2h,l,p). This is unlikely to have been affected by the higher density of biomass present in this region (e.g., more biomass rather than a higher ^{32}P concentration), as this trend would have also been observed in P-sufficient plants as they had higher biomass compared to P-deficient plants (Figure S2h,l,p).

After the 7 days application, there was evidence of leaf scorching in P-sufficient Gairdner and Macquarie (Figure 7e,g) and also in P-deficient Gairdner, Macquarie and Westminster (Figure 7I,k,o). As the foliar-applied solution was at pH 6, this was probably due to the solution concentration rather than the acidity. ^{32}P was translocated and accumulated in younger leaves and tillers in both P-sufficient and P-deficient plants (Figure 7). However, P-sufficient cultivars translocated and accumulated ^{32}P to multiple leaves and tillers, while translocation and accumulation were largely limited to the newly emerging leaf in P-deficient cultivars. When comparing P-sufficient cultivars, Gairdner translocated and accumulated more ^{32}P in older leaves (Figure 7e,f). Macquarie translocated less ^{32}P to untreated shoots (Figure 7j), while Flagship showed

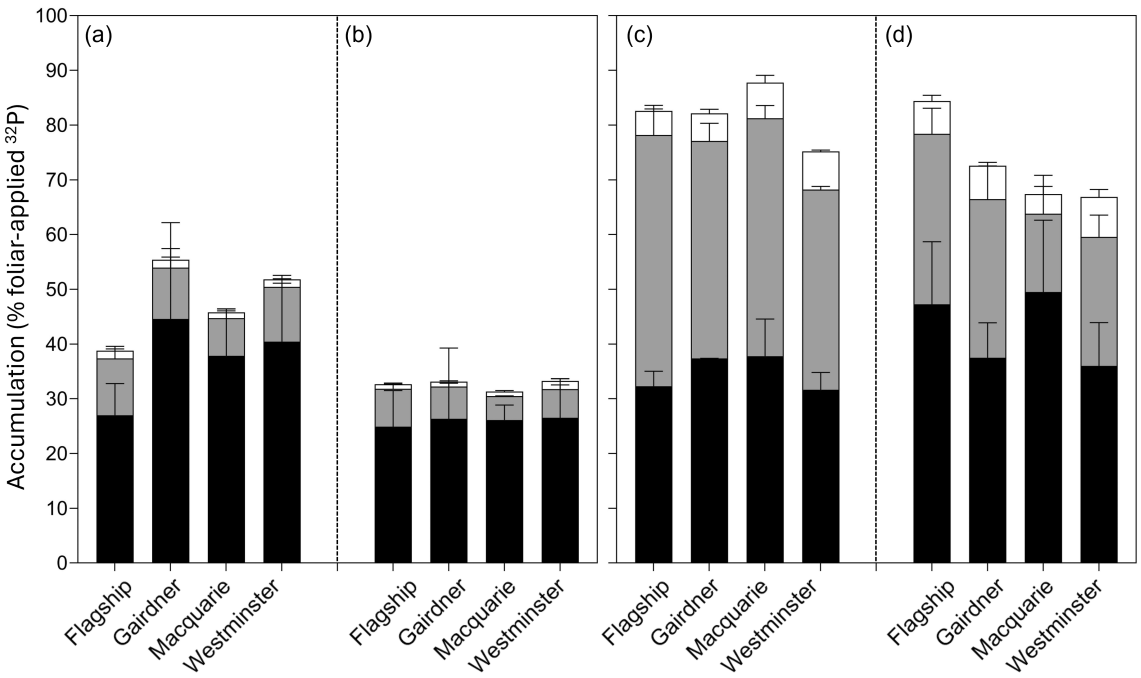


FIGURE 6 | Translocation and accumulation of foliar-applied ^{32}P after 48 h (a, b) or 7days (c, d) in the treated leaf (black bars), untreated shoots (grey bars) and roots (white bars) of P-sufficient (a, c) or P-deficient (b, d) barley cultivars. Percentages were calculated as a proportion of total applied foliar ^{32}P. Error bars show SE ($n = 3$).

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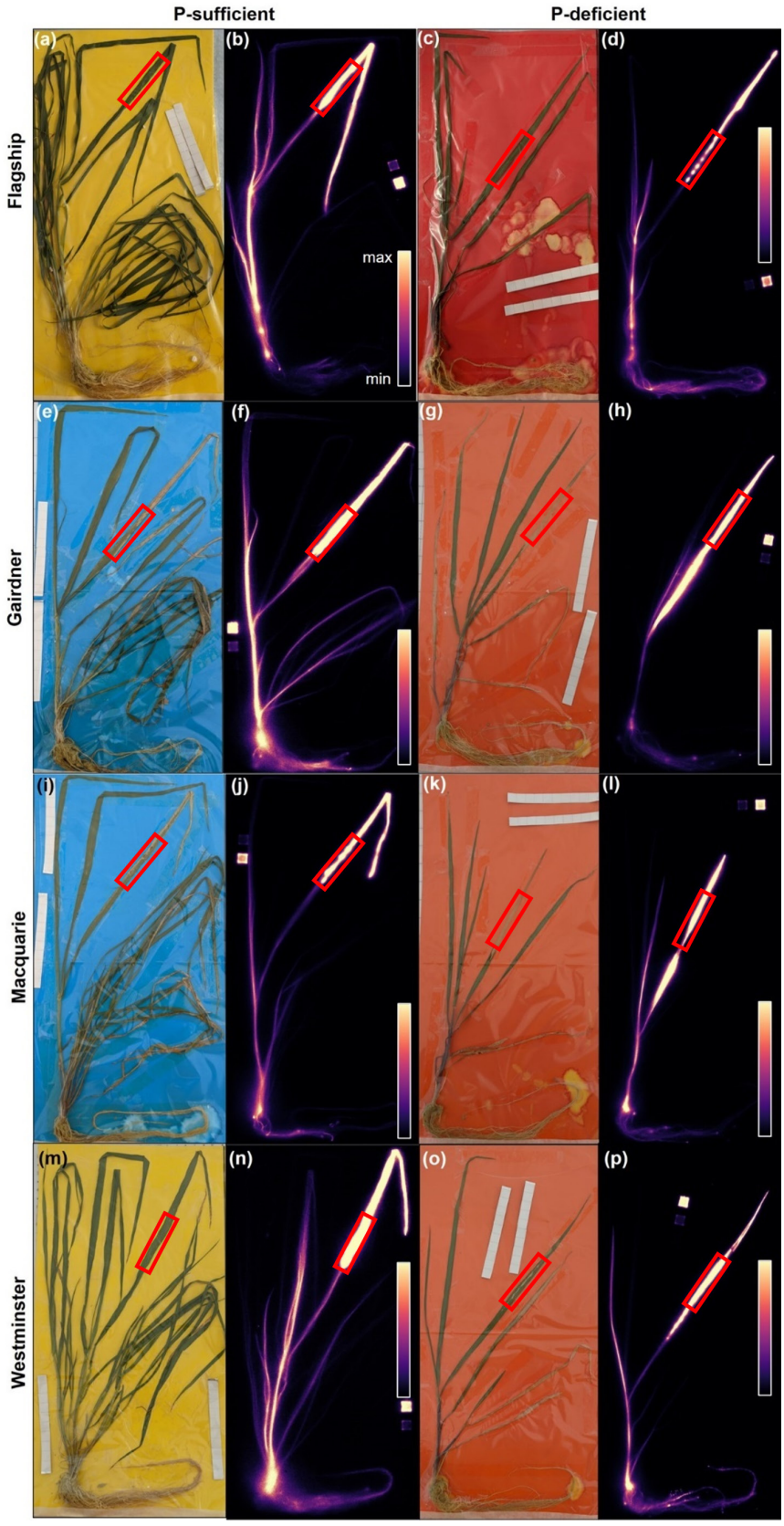


FIGURE 7 | ^{32}P accumulation in dried whole plants of barley cultivars visualised via autoradiography following a 7 days foliar application period. Scale bar shows dark to brighter colours indicate increasing concentrations of ^{32}P. Colour photographs of each cultivar are provided for each autoradiograph for anatomical reference. Autoradiographs were adjusted to the same threshold signal intensity using Fiji ImageJ and the square ^{32}P standards. Red boxes indicate where foliar-applied ^{32}P was applied directly to the adaxial leaf surface.

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little translocation and accumulation in the roots (Figure 7b). Among P-deficient cultivars, Gairdner translocated and accumulated less ^{32}P to the roots (Figure 7k,l). Similarly, P-sufficient Gairdner, Macquarie and Westminster and P-deficient Macquarie and Westminster appear to show high ^{32}P accumulation in plant crowns, which was unlikely to be an artefact of biomass thickness as it was not observed to occur in all plants (Figure 7f,j,l,n,p).

4 | Discussion

While trichome and stomatal densities, as well as the epidermal cuticle and cell wall thickness, varied between cultivars and due to plant P status, this did not translate to significant differences in foliar-applied P absorption in plants after 48 h. After 7 days, P-deficient plants were found to absorb significantly less foliar-applied P compared to P-sufficient plants, and autoradiography images also showed that translocation and accumulation of the foliar-absorbed ^{32}P were largely limited to the newly emerging leaf. However, after 7 days, all cultivars were found to have relatively high foliar absorption (> 65% of foliar-applied P) regardless of plant P status or any identified differences in leaf surface morphology.

4.1 | Leaf Surface Properties as Affected by Plant P Status

Under P-sufficient conditions, the Macquarie cultivar had the highest trichome densities, and Flagship had the lowest, while Flagship had a thicker epidermis cuticle and cell wall than Macquarie. Under P-deficient conditions, Flagship had significantly higher trichome densities than other cultivars and higher stomatal densities than Gairdner, while Macquarie and Westminster had thicker epidermis cell walls than Gairdner. Overall, Flagship responded to P deficiency in a way that should not decrease P absorption, as trichome and stomatal density both increased and the cuticle and epidermal cell wall thickness did not change (Table 2). In contrast, Macquarie showed the most changes in leaf surface properties that could negatively affect P absorption in P-deficient plants. In this case, trichome density and stomatal density significantly decreased, and cuticle and cell wall thickness significantly increased. P deficiency has been reported to have variable effects on leaf surface morphology and properties (e.g., P-deficiency reduced stomatal and trichome densities in wheat but had minor or no effect on stomatal densities in P-deficient maize or barley) (Fernández et al. 2014; Arsic et al. 2022; Henningsen, Görlach, et al. 2023). There was also no difference in foliar-applied P absorption reported between four maize cultivars (Henningsen, Görlach, et al. 2023).

4.2 | Plant P Status Effects on Leaf Surface Properties and Foliar-Applied P Absorption

P-deficiency decreased plant biomass production, total P tissue concentrations and increased root-to-shoot ratios for all cultivars. While P-deficiency led to an overall decrease in trichome and stomatal densities and an increase in the thickness of the cuticle and epidermal cell wall thickness, changes were specific to each cultivar. Macquarie was most affected by P-deficiency, where both trichome and stomatal densities decreased, whereas the epidermal cuticle and cell wall thickness increased. Conversely, trichome densities increased due to P-deficiency in Flagship, where P uptake was the least impaired by P-deficiency among all cultivars (Table 2). Flagship was developed for warmer, drier central Australian regions as opposed to moderate-high rainfall areas like Gairdner, Macquarie, and Westminster. Gairdner showed no significant differences in leaf surface properties due to plant P status. Furthermore, P-deficiency increased the epidermal cuticle and cell wall thickness in Macquarie and Westminster. This could possibly be due to plants adopting a conservative approach toward resource use and protecting existing organs in a nutrient-scarce environment, where the increased thickness of the epidermis cuticle and cell wall could minimise pathogenic infection, irradiation damage and water loss (Yeats and Rose 2013).

After 7 days, P-sufficient plants absorbed more foliar-applied P than P-deficient plants, although this effect could not be statistically attributed to specific groups of cultivars. A suite of leaf surface modifications may have led to lower P absorption, suggesting that multiple uptake processes may occur simultaneously. Between 66% and 84% of foliar-applied P was absorbed by P-deficient plants after 7 days. This contrasts a study in wheat by Fernández et al. (2014), who showed that severely P-deficient wheat plants with comparable tissue P concentrations (~800 mg P kg^{-1}) failed to absorb any foliar-applied radiolabelled P within 48 h. While the adhesion of water droplets was higher in P-deficient plants, the wheat cultivar (Axe) had a thinner cuticle and much lower stomata and trichome densities compared to barley cultivars in this study. Barley and wheat may have different leaf cuticle permeabilities, or this lower density of trichomes and stomata may have had a greater impact on foliar-applied P absorption. Additionally, the Fernández et al. (2014) study used monoammonium phosphate (MAP) while DAP was used in this study; the two phosphate salts have different point of deliquescence values (POD) and likely points of efflorescence (POE) values. POD values indicate the RH value when solutes become liquid due to water absorption from the atmosphere, while the POE indicates the efflorescence RH when a previously saturated solution crystallises to become solid; therefore, the POE determines the ERH when the droplet dries, and POD determines the DRH when the foliar-applied droplet is rehydrated (at a specific temperature) (Fernández et al. 2020). While POE values have not yet been reported and only the DRH values for MAP and DAP are reported at 30°C (91.6% and 82.5%, respectively), the lower POD value of DAP may lead to more P becoming available in solution and thus facilitating absorption across the leaf surface (Sharma and Patel 2000). However, these values need to be validated for the appropriate range of temperatures in this experiment (20°C–25°C), and the POE values were calculated to consider the role of these processes in foliar absorption.

4.3 | Pathways for Foliar P Absorption

In this study, quantitative foliar-applied P absorption could not be linked to a specific leaf surface morphological structure or

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property. However, the results suggest that multiple absorption pathways may play a role in P absorption. A similar study in maize reported that changes in leaf stomatal densities and cuticle composition due to plant P status could not be specifically linked to foliar-applied P absorption (Henningsen, Görlach, et al. 2023). While leaf wettability was reported to be the major driver for foliar-applied P absorption in P-deficient maize, all P-sufficient and P-deficient barley plants were wettable in this study due to the addition of a surfactant (Henningsen, Görlach, et al. 2023).

Trichomes, fibre cells, stomata and the cuticle may all contribute to the net absorption of foliar-applied P and subsequent accumulation in plant tissues. Trichomes and/or fibre cells occurring on vein ridges were the dominant zones for foliar P absorption in barley and tomato leaves, while there was an indication of cuticular and stomatal penetration (Arsic et al. 2020, 2022; Henningsen, Bahamonde, et al. 2023). While there was a high density of smaller trichomes occurring in interveinal regions in P-sufficient Macquarie and P-deficient Flagship, leaf cross-sections in this study revealed no such sub-epidermal structures below these types of trichomes. This raises questions about whether these trichomes can facilitate ion absorption and translocation into the leaf tissue without the underlying fibre cell and bundle sheath extension structures. If different types of trichomes perform different functions, their densities may not be correlated with foliar absorption. However, this speculation requires further experimental investigation using high-resolution bioimaging methods (e.g., LA-ICP-MS or x-ray fluorescence microscopy) to determine whether different trichome structures may or may not be associated with foliar P absorption.

It may be difficult to correlate stomatal densities with foliar nutrient absorption because the variation in stomatal density between cultivars was not significant, and P-deficiency only caused small changes in the cultivars tested (i.e., only Macquarie showed significant variation in densities). The stomatal pathway can also be highly variable; less than 10% of stomata were observed to facilitate the penetration of 43 nm hydrophilic polystyrene particles (Eichert et al. 2008). The process of 'hydraulic activation' of stomates involves hygroscopic particles accumulating in the pore, establishing an aqueous continuum between the cuticle and the substomatal cavity to allow ion penetration (Burkhardt 2010). This may depend on individual microenvironments around each stomate, such as deposited particles, microbial growth, or the structure and composition of the guard cell or pore wall cuticles (Burkhardt et al. 2012). Lower absorption of foliar-applied P in P-deficient wheat was suggested to be due to 'stomatal malfunctioning' as seen in the reduced absorption of boron (B) in B-deficient soybean, possibly due to shrunken and closed stomata, or limited stomatal aperture in response to stress (P-deficient wheat plants did not absorb irrigation water during pot trials) (Will et al. 2011; Fernández et al. 2014). A limitation of this study is that it did not measure stomatal performance and subsequent effects on leaf porosity and absorption (e.g., malformed guard cells, changes in stomatal size or aperture size, stomatal conductance, frequency of stomatal solute penetration), which may have affected foliar-applied P absorption as observed in the study on B-deficient soybean. This factor should be investigated in future studies by utilizing high-resolution microscopy, bioimaging techniques, and porometers.

While the literature shows that thin cuticles generally tend to be better and more efficient water barriers than thick cuticles, and water permeability does not appear to be affected by cuticle thickness or wax coverage (Becker et al. 1986; Schonherr and Riederer 1989; Buchholz et al. 1998; Schreiber 2009), some studies have linked solute absorption to changes in thickness. Foliar absorption of Zn, Mn and Fe was negatively correlated to epidermal cuticle and cell wall thickness, where an increased thickness led to a decrease in foliar absorption for tomato and sunflower leaves (Li et al. 2017). Similarly, in our previous study, less P penetrated into the leaves of P-deficient barley plants compared to P-sufficient plants over 6 h, where P-deficient plants had thicker cuticles and epidermal cell walls and a lower abundance of polysaccharides (Arsic et al. 2022). A similar result was observed in the current study, even though cuticle and epidermal cell thickness was only significantly increased in Macquarie and Westminster (Table 2). However, a major limitation of this study is the lack of chemical composition analysis for the adaxial leaf surface, which was necessary to distinguish whether physical or chemical properties under different nutrient conditions affected foliar-P absorption (as reported in Li et al. 2017). Analyses such as the chemical composition of the cuticle and epidermal cell wall (FTIR) and ultrastructure properties (TEM) should be included in future work (Henningsen, Görlach, et al. 2023).

4.4 | Translocation and Accumulation of Foliar-Applied P

Most of the absorbed foliar-applied P accumulated in the treated leaf after 48 h, whereas a majority of the P had been translocated and accumulated in other leaves and tillers after 7 days. Phosphorus is a highly mobile nutrient that is rapidly translocated throughout the plant, and foliar-applied P has been shown to reach newly emerging leaves within 7 days (Koontz and Biddulph 1957; Arsic et al. 2020). While translocation to the roots and P accumulation increased after the 7 days foliar application, this represented a small proportion of the total foliar-applied P absorbed. Other studies using foliar-applied radioisotopes in wheat grown to maturity showed that more of the foliar-applied P was translocated to the grain and other plant parts when applied at flag leaf emergence rather than early tillering (Peirce et al. 2019). Solution formulation (adjuvant and accompanying cation) was shown to affect translocation from the treated leaf; for example, phosphoric acid applications showed high absorption but lower translocation caused by low pH leaf scorching, which could have also been the case in P-deficient Macquarie (McBeath et al. 2020). P-deficient maize showed that while there was a rapid absorption and translocation of foliar-applied P, biomass effects were variable at flowering but not at harvest, supporting other studies that compare the high initial absorption with the transient nature of single applications of foliar-applied P on biomass (Arsic et al. 2020; Görlach, Henningsen, et al. 2021; Görlach, Sagervanshi, et al. 2021).

Autoradiography allowed for more subtle discernment regarding foliar-applied P accumulation and partitioning within the plant. As the quantitative liquid scintillation method is a mass-averaged method, for example, all untreated leaves and tillers were pooled together, autoradiography showed that P-sufficient plants were more effective at distributing foliar-applied P

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throughout the shoots. P-deficient plants largely translocated and accumulated foliar-applied P in the newly emerging leaf. Furthermore, P deficiency affected translocation to the roots differently amongst cultivars; P-deficient Flagship accumulated more foliar-applied P to the roots, while P-deficient Gairdner accumulated less. These trends could provide information as to the potentially different P absorption or utilization efficiencies for foliar applications amongst cultivars, although this would need to be confirmed by harvesting plants at maturity.

In conclusion, the specific pathways for foliar-applied P absorption across the leaf surface remain elusive. Barley cultivars differed in their trichome and stomatal densities, as well as epidermal cuticle and cell wall thickness, under both P-sufficient and P-deficient conditions. However, while P-deficient plants absorbed less foliar-applied P after 7 days, no dominant changes in leaf surface properties or pathways could be linked to this decrease. Instead, a number of leaf properties likely contributed to this effect. Furthermore, differences in hydraulic activation may be a factor and should be considered in future studies, where plants may have similar stomatal densities but differ in the extent to which these pathways are activated. Despite this, all plants absorbed relatively high proportions of foliar-applied P (> 65%) regardless of cultivar or plant P status, indicating that foliar fertilization may be a useful top-up strategy. Substantial translocation was observed to untreated shoots in all cultivars except for P-deficient Macquarie, although autoradiographs showed that translocation and accumulation were largely limited to newly emerging leaves when plants were P-deficient. Future studies should consider stomatal functioning in addition to density, the potential difference in function between trichomes associated with leaf veins or occurring in interveinal regions, and investigate the effects of P-deficiency on the chemical composition and ultrastructures of the leaf surface cuticle and epidermal cell wall.

Author Contributions

All authors contributed to the experimental design. E.L. and C.L.D. supervised the overall research plan. M.A. performed plant experiments with assistance from G.B. and C.L.D. N.R.H., T.C., E.L., C.L.D. and M.A. designed and optimised foliar ^{32}P applications for autoradiography. N.R.H. performed all autoradiography imaging, and N.R.H. and M.A. did the image processing. M.A. wrote the manuscript and revised it together with N.R.H., T.C., E.L., C.L.D., G.B., J.K.S., D.P.P. and S.H. All authors have read and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Datasets generated during and/or analysed during the current study can be made available upon reasonable request to the corresponding author.

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