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RESEARCH ARTICLE

Arbuscular mycorrhizal fungal inoculation increases the bioavailability of zinc and iron in wheat grain

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Social Impact Statement

Bread wheat is an important crop that supplies calories and nutrients to the global population. Enhancing the concentrations and bioavailability of essential micronutrients in wheat is crucial for human nutrition, and we investigated whether arbuscular mycorrhizal (AM) fungi can contribute to the biofortification of wheat. In this study, AM fungal inoculation increased the Zn uptake of Australian bread wheat, without elevating phytate levels, which led to improved Zn and Fe bioavailability compared to non-AM plants. AM fungi could contribute to the biofortification of bread wheat but must be considered within the broader agronomic context.

Summary

- Bread wheat, the world's second major food crop, is crucial for global nutrition. Enhancing Zn and Fe bioavailability in wheat grain can combat human nutritional deficiencies. There is demonstrated potential for AM fungi to support these goals through increased uptake of Zn and Fe into wheat. However, AM fungi can also increase P uptake, leading to higher phytic acid levels in the grain, which can hinder Zn and Fe absorption in the digestive system.
- Eight Australian wheat varieties were grown with or without AM fungi (*Rhizophagus irregularis*) and two soil P treatments (addition of 0 or 25 mg P kg⁻¹ soil) in a controlled growth environment. At maturity, plants were harvested and analysed for grain biomass, nutrition, phytate and 2D spatial elemental distribution within grain using X-ray Fluorescence Microscopy.
- Our results indicated that the AM-colonised plants had greater grain biomass and accumulated greater amounts of P and Zn in whole grain and Zn in the aleurone layer, but not Fe. Increased P did not raise phytate levels, leading to overall higher Zn and Fe bioavailability in AM-inoculated plants compared to non-inoculated controls.
- AM fungal inoculation could be a promising strategy for producing wheat grain with higher micronutrient bioavailability for human nutrition, without compromising agronomic practices (P fertiliser application) or yield targets.

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KEYWORDS

arbuscular mycorrhizal fungi, iron, micronutrient bioavailability, phytate, wheat, zinc

1 | INTRODUCTION

Future global population growth will drive higher demand for food, requiring food in greater quantity and of improved nutritional quality (Tilman et al., 2011). Deficiencies in micronutrients, especially of zinc (Zn) and iron (Fe), are widespread in the human populations of high-, low- and middle-income countries, affecting approximately 30% (Zn) and 60% (Fe) of the global population, respectively (White & Broadley, 2009). Human Zn deficiency affects normal development during pregnancy, childhood and adolescence; skin and hair; and leads to diarrhoea and diseases (Yanagisawa, 2004). The most common outcome of human Fe deficiency is iron deficiency anaemia, which is associated with impaired immune function and cognitive development and causes higher mortality of mothers and children at birth (Cakmak, 2008; Soliman et al., 2019). In low- and middle-income countries, cereal-based diets with low meat intake are common and lead directly to Zn and Fe deficiencies (Çakmak et al., 1999; Gupta et al., 2020). Conversely, in high-income countries, although meat serves as the primary source of dietary Zn and Fe, cereal products still provide around 20–25% of dietary Zn (Baghurst et al., 2000; Gibson et al., 2001). Bread wheat is the second major food crop produced in the world, as it is a staple food for a significant portion of the world's population (Statista, 2024). Wheat products, specifically bread, contribute up to 17% of the dietary intakes of Zn and Fe, respectively (Bates et al., 2014). Hence, enhancing the bioavailability of Zn and Fe in wheat grains holds immense potential for combating nutritional deficiencies (Cakmak & Kutman, 2018; Stangoulis & Knez, 2022).

Bioavailability is defined as the proportion of a nutrient that can be absorbed into the bloodstream after ingestion for utilisation by the body (Carbonell-Capella et al., 2014; Clemens, 2014). In cereal-based food products, the bioavailability of Zn and Fe is affected by factors such as chemical speciation (how the mineral is chemically bound) and the localisation of Zn and Fe within grain structures (i.e., aleurone layer, starchy endosperm and embryo). The majority of plant phosphorus (P) is stored in cereal grains as phytic acid (PA or phytate, myo-inositol hexaphosphate) (Gupta et al., 2015). Phytate is one of the main inhibitors of the absorption of Zn and Fe in the gut (Lebert et al., 2022). In plants, phytase enzymes can break down PA to release P and other complex elements, which support the early growth of the plant. However, phytase does not exist in the human body, so the presence of PA in cereal products directly decreases the bioavailability of Zn, Fe and other cations, thus reducing the nutritional quality of cereal-based foods (Gupta et al., 2015; Oatway et al., 2001). Therefore, PA is considered an anti-nutritional agent in cereal products that may exacerbate micronutrient deficiencies in humans (Gupta et al., 2015; Samtiya et al., 2020).

International efforts to improve the bioavailability of micronutrients, termed biofortification, address the issue from two angles: increasing Zn and Fe uptake/translocation to the grain and reducing

phytate accumulation (Cakmak et al., 2010; Maganti et al., 2020; Pfeiffer & McClafferty, 2007). Crop biofortification has been accomplished through traditional breeding methods as well as the application of genetic engineering techniques (Stangoulis et al., 2007). For example, *OsNAS2*, a chelator of Zn and Fe, was overexpressed in rice and wheat and resulted in a 1.5 × and 2.0 × increase in Zn and Fe concentration, respectively, in rice (Johnson et al., 2011), and a 1.6 × and 1.5 × increase in Zn and Fe, respectively, in wheat (Beasley et al., 2019).

Arbuscular mycorrhizal (AM) fungi are soil fungi that colonise roots and can affect crop growth and yield via the uptake of nutrients, especially P (Gianinazzi et al., 1990). AM fungi can colonise more than 80% of terrestrial plant species (Smith & Read, 2010), including almost all economically important crops, including cereals (e.g. barley, rice, sorghum and wheat) (Al-Karaki et al., 2004; Baon et al., 1993; Raju et al., 1990; Solaiman & Hirata, 1995), legumes and many horticultural crops (Ibijbijen et al., 1996; Maronek et al., 1981). A meta-analysis on AM fungal impact on Zn uptake reported that AM inoculation increased the Zn concentration in shoots, edible fruits and edible seeds of wheat, corn and rice plants (Lehmann et al., 2014).

Although AM fungi have the potential to increase plant Zn and Fe uptake, thereby theoretically contributing to biofortification (Pellegrino & Bedini, 2014), the potential caveat is that phytate also accumulates because of the greater P supply via the mycorrhizal pathway of uptake. Studies in winter wheat and sorghum found that AM fungal inoculation improved the Zn and/or Fe bioavailability in grains by increasing the Zn and Fe concentration while having slight or no effect on grain phytate content (Ma et al., 2019; Watts-Williams et al., 2022). On the other hand, Ryan et al. (2008) found that AM fungal inoculation increased grain phytate content but had a negligible effect on Zn and Fe bioavailability in bread wheat. In this study, we investigated the effects of *R. irregularis* inoculation in interaction with soil P fertilisation on eight Australian bread wheat varieties and addressed the following research questions:

- i. Does AM fungal inoculation affect the concentrations of human essential micronutrients in wheat grain?
- ii. Is the concentration of zinc in the aleurone layer of wheat different from the whole grain?
- iii. How does soil P availability interact with AM fungal colonisation and phytate accumulation in wheat grain?

2 | METHODS

2.1 | Soils, plants and fungi

The soil used was clay loam collected from the Gawler River region of South Australia (Clay & Mineral Sales Pty Ltd, Adelaide). This is an

alkaline soil, which is common throughout Australian cropping regions and accounts for 80% of soils in the cereal-growing regions of South Australia (GRDC, 2010). The soil was firstly air-dried and then sieved to less than 2 mm to homogenise and eliminate any large debris. The soil was then autoclaved twice with >24 hours between runs, and fine sand was steam sterilised and air-dried before mixing with the prepared soil in a ratio of 1:1 w/w using a cement mixer. This mixture was referred to as “soil” thereafter. The soil had a pH of 8.49 (1:5 water) and a plant-available (Colwell) P concentration of 8 mg P kg⁻¹. The concentrations of DTPA-extractable Zn and Fe were 0.23 mg kg⁻¹ and 7.9 mg kg⁻¹, respectively (Eurofins APAL). Soil P treatments were applied by adding KH₂PO₄ solution and mixed thoroughly through the soil at concentrations of 0 and 25 mg P kg⁻¹ soil, resulting in two levels of plant-available (Colwell) P concentrations: 8.0 mg P kg⁻¹ (referred to as Low P) and 23 mg P kg⁻¹ (referred to as High P). The AM treatment was inoculated with the AM fungus *R. irregularis* from a commercial product (Start Up Ultra, Microbe Smart Pty Ltd) at a rate of 1.5 g kg⁻¹ soil (corresponding to approximately 800 spores g⁻¹) and then mixed thoroughly. Plastic, free-draining pots with a volume of 0.8 L were filled with the prepared soils. The experiment was fully factorial for each P, AM fungal and Variety treatment, for a total of 32 treatments, each with six biological replicates, giving a total of 192 pots.

Eight bread wheat varieties cultivated in Australia were selected, namely, Calibre, Gladius, Mace, Rockstar, Scepter, Spitfire, Trojan and Vixen. Seeds were firstly surface-sterilised with 10% sodium hypochlorite solution (NaClO) for 10 minutes, then washed and rinsed with reverse osmosis (RO) water three times. The seeds were placed onto a wet paper towel, enclosed in a plastic container and kept in the dark at 28°C for two days. After that, the seeds were moved to the lab bench at room temperature for two days. Once the shoots emerged, the seedlings were transplanted to the prepared pots, with two plants per pot. The growth of two plants per pot may have implications for interpreting the data, due to potential competition for resources between the two plants or connection via a common mycorrhizal network that may have influenced plant nutrient uptake and allocation.

2.2 | Plant growth and harvest

Plants were grown in a controlled environment room on the University of Adelaide's Waite Campus, Australia. The conditions were set at 24/20°C day/night temperature and 14/10 hour day/night photoperiod during the growing period. Plants were watered every second or third day with RO water to 10% of the soil weight. Plants were supplemented with 10 ml of full-strength modified Long-Ashton solution (P omitted [Cavagnaro et al., 2010]) once every two weeks during the first month after planting and then every 10 days subsequent to that. Plants were fertilised with 10 mg N per pot in the form of NH₄NO₃ solution at 30 and 45 days after planting (DAP).

Fresh root subsamples for AM colonisation analysis were taken seven days before the destructive harvest by using small soil cores

(1.5 cm diameter and 11 cm length). The subsample of roots was washed to remove soil before being placed in tissue cassettes and submerged in a 50% ethanol solution for at least 24 hours. All plants were destructively harvested between 81 and 95 DAP depending on when the different varieties reached maturity. One seed was taken from each plant in the pot, giving a total of two seeds per biological replicate and stored at 4°C for synchrotron X-ray fluorescence microscopy analysis. The aboveground biomass (shoots and grain) was cut at the soil level and weighed to determine the total fresh shoot biomass. Photos of representative plants from the Low P treatment, comparing *R. irregularis* inoculation to no AM fungal inoculation, are shown in Figure S1). The aboveground biomass was then dried at 50°C in an oven for at least 48 hours prior to the determination of shoot dry weight. Once the shoot dry weight was determined, the grain was separated by using a manual threshing board and the grain dry weight was recorded.

2.3 | Sample analysis

The fresh root subsamples were rinsed to remove the ethanol and placed into a 10% potassium hydroxide (KOH) (w/v) solution for clearing at room temperature for seven days. Following this, the samples were rinsed well and then stained in a 5% ink in vinegar solution (Vierheilg et al., 1998) and then de-stained in acidified RO water for at least 24 hours before being stored in 50% glycerol solution. The samples were then quantified for percentage root length colonised by AM fungi under a stereomicroscope using the gridline intersect method (Giovannetti & Mosse, 1980).

A Geno/Grinder 2010 (SPEX SamplePrep) was used to homogenise the grain samples before being sub-sampled for acid digestion. Subsequently, the weighed grain subsamples were cold digested overnight using a 4:1 (v/v) mix of nitric acid (HNO₃) and hydrogen peroxide (H₂O₂), followed by a ramping hot digest programme in a heat block at 80°C for 30 minutes and then 125°C for 150 minutes (Zarcinas et al., 1987). The acid-digested samples were then diluted with RO water and filtered before the determination of P, Zn and Fe analysis by inductively coupled plasma optical emission spectroscopy (PerkinElmer - Optical Emission Spectrometer|Avio 200 ICP).

The grain phytic acid content was determined by using a commercial phytic acid/total phosphorus assay kit (Megazyme, Ireland). A ~250 mg sub-sample of the homogenised grain sample was weighed into 10 ml tubes and then extracted with 5 ml of HCl (0.66 M) on an orbital shaker (40 rpm) for 16 h at room temperature. The following day, the manufacturer's protocol was followed and the PA content of samples was determined by spectrophotometer at 655 nm wavelength. To estimate the Zn and Fe bioavailability in the grain, the molar concentrations of PA, Zn and Fe were calculated. The molar ratio of PA to Zn, or to Fe, was used as a proxy for bioavailability (Reddy, 2001). When the ratio of PA to Zn is <5, between 5 and 15, or >15, it signifies high, medium and low bioavailability of Zn, respectively (Gibson, 2006). Likewise, a PA: Fe < 1 or >1 indicates high and low Fe bioavailability (Hurrell & Egli, 2010).

2.4 | Grain elemental localisation: Synchrotron X-ray Fluorescence Microscopy (XFM)

Wheat grain sections were prepared by aligning them vertically using a 3D-printed scaffold, which consisted of a plastic grid in which the grains were superglued (Loctite 401). One grain was taken from each pot for XFM analysis, and three biological replicates were analysed per treatment. This assemblage was then embedded in resin (Araldite GY 191 Huntsman) and, after curing, sectioned using a diamond blade. The embedded samples were glued to quartz slides, and a thin section ($\approx 180 \mu\text{m}$) of the resin-embedded grain was prepared by cutting with a diamond blade and polishing with lapping film (Starcke 991A silicon carbide paper 1,200 grit and other grit sizes) (Doolette et al., 2020). After that, the section was mounted on a sample stage at the XFM Beamline at the Australian Synchrotron (ANSTO) in Melbourne, Victoria. The incident beam was set at 15.8 keV using a Si (111) monochromator and focused to approximately $2 \mu\text{m} \times 10 \mu\text{m}$ using Kirkpatrick-Baez mirrors (Paterson et al., 2011). Elemental maps were generated by collecting the X-ray fluorescence signal emitted by the sample using a 384-element Maia detector in backscatter geometry. The samples were scanned on the fly in the horizontal direction with discrete steps in the vertical direction. The sampling interval was 0.02 mm with a vertical step of 0.02 mm, and the dwell time for each pixel was 6.6 ms.

After collecting the elemental maps, Zn K-edge X-ray Near Edge Spectroscopy (XANES) data were generated by selecting a line across grain sections of interest (including the crease). These lines were scanned at 109 energies (from 9,609 to 9,843 eV) across the Zn K-edge with a step size of 0.005 mm and a dwell time per pixel of 10.0 ms. The parts of the line scans corresponding to the aleurone layer (both at the periphery of the grain and in the crease) were averaged per grain to obtain Zn XANES spectra of this tissue. Standards of Zn bound to histidine, cysteine, citrate, phytate and polygalacturonic acid were collected in the same way.

The XFM data were analysed using GeoPIXE (Ryan, 2000; Ryan & Jamieson, 1993). The analyses of the elemental maps were conducted with an ImageJ-based pipeline as detailed in Ren et al. (2023). This approach allowed semi-automatic extraction of average concentrations of elements in whole grains, endosperm and aleurone layers, and the XANES data were analysed by linear combination fitting using the Athena software.

2.5 | Statistical analysis

Data were analysed using R statistical software v. 4.1.1 (Team, 2020). A three-way analysis of variance (ANOVA) was conducted to analyse grain dry weight, P, Zn and Fe concentration and bioavailability, and phytate content, with *Variety*, *P* and *Mycorrhiza* as treatment factors. For % AM colonisation and % MGBR, a two-way ANOVA was performed, incorporating *P* and *Variety* as treatment factors. Following ANOVA, if there was a significant interaction term or main effect, Tukey's honestly significant difference (HSD) *post hoc* test was used to compare treatment means.

3 | RESULTS

3.1 | AM colonisation and grain biomass

All wheat varieties were well colonised by the AM fungus (*R. irregularis*). Root AM colonisation was greater in the Low P treatment (mean 66.05% root length colonised) than in the High P treatment (mean 40.28% root length colonised) in all varieties (Figure 1). Additionally, when Variety was considered as the sole factor, Scepter and Vixen had lower root colonisation than other varieties. No AM colonisation was detected in any non-inoculated plants. Additionally, there was no significant interaction between P treatment and Variety (see Table S1 for ANOVA outcome).

There was no significant three-way interaction observed for grain dry weight (GDW), but it was influenced by the main effects of soil P treatment and AM fungal inoculation. The GDW was greater in the High P treatment compared to the Low P treatment and pooling wheat varieties, and the values ranged from 1.49 g (Spitfire non-AM, Low P) to 2.15 g (Scepter, AM, High P) (Figure 2). AM inoculation increased the grain biomass of wheat varieties only at the Low soil P concentrations, especially in Scepter and Spitfire plants, by 7–10% compared to the respective non-inoculated plants (Figure S2a).

3.2 | Whole grain and aleurone layer nutrient concentrations

The concentrations of key nutrients (P, Zn and Fe) were analysed in grain samples to determine how AM colonisation and soil P fertilisation affect nutrient uptake in different wheat varieties. A significant three-way interaction was found to affect whole grain P concentration (see Table S2 for ANOVA outcomes). With low soil P fertiliser, many wheat varieties showed higher whole grain P concentrations in

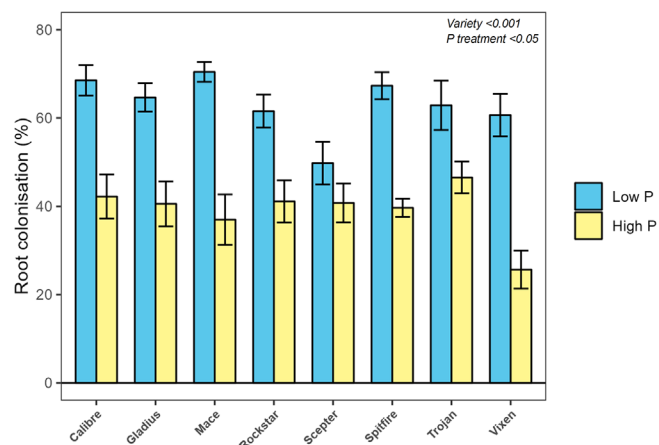


FIGURE 1 Arbuscular mycorrhizal root colonisation of eight bread wheat varieties inoculated with the arbuscular mycorrhizal fungus (AMF) *R. irregularis* and grown at low soil phosphorus (P) (blue bars) and high soil P fertiliser (yellow bars). Values are mean \pm SEM, $n = 6$.

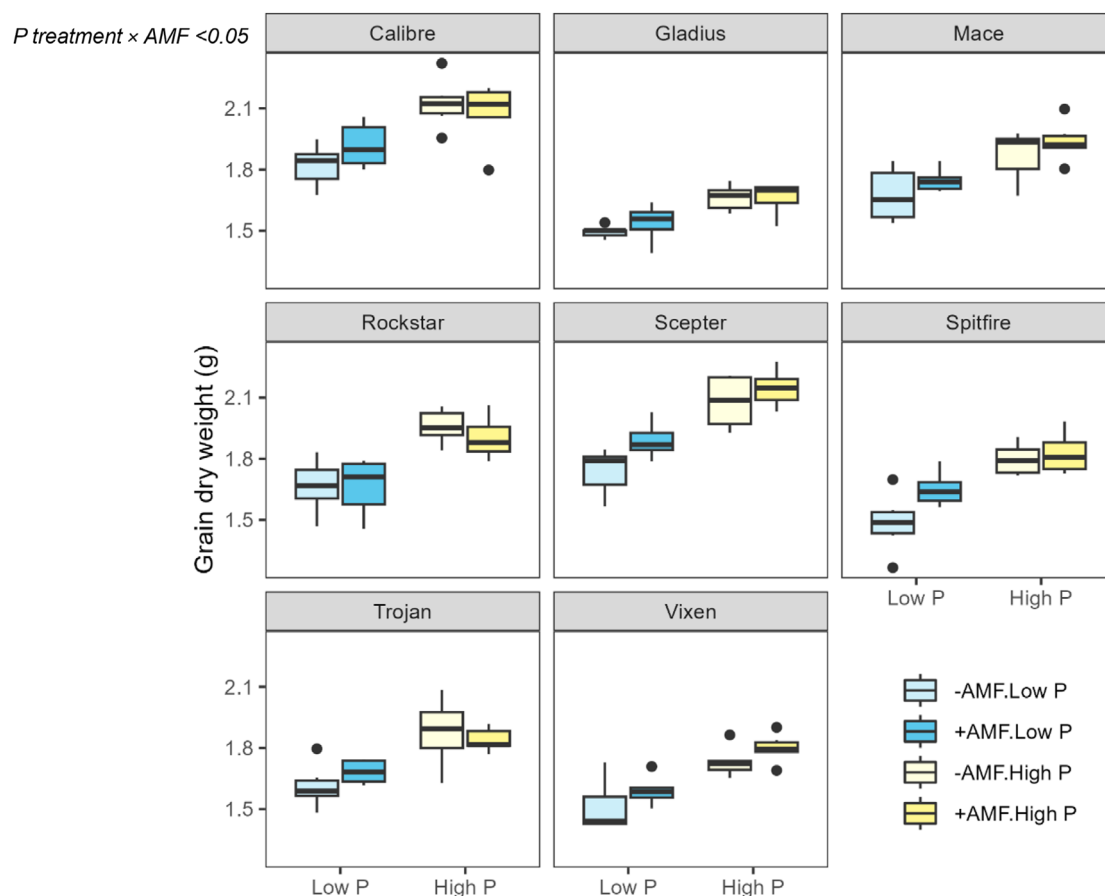


FIGURE 2 Grain dry weights of eight wheat varieties inoculated with the arbuscular mycorrhizal fungus (AMF) *R. irregularis* (blue and yellow bars) or mock-inoculated (light-blue and light-yellow bars) and grown at low and high soil phosphorus (P) fertiliser. Thick black lines on the box-and-whisker plots are median values, $n = 6$.

AM-inoculated plants compared to non-AM plants, except for the Gladius and Spitfire. Additionally, at high soil P levels, AM inoculation increased whole grain P concentration in the Gladius and Trojan varieties (Figure S2b).

The whole grain Zn concentrations were influenced by P treatment, AM fungal inoculation and Variety, and there was no significant interaction effect between these factors (Figure 3a). For five varieties (Calibre, Mace, Rockstar, Scepter and Trojan), AM-inoculated plants had higher whole grain Zn concentrations in both Low P and High P soils. In the Gladius and Spitfire varieties, AM inoculation increased whole grain Zn concentrations only in High P treatment, while in Vixen, this effect was observed under Low P treatment (Figure 3a). Notably, Gladius plants had significantly higher whole grain Zn concentration compared to other varieties, particularly under low P conditions, with a mean of $19.26 \text{ mg Zn kg}^{-1}$.

Whole grain Fe concentration was affected by the three-way interaction, but there was no significant difference between AM and non-AM plants across all varieties under Low and High P treatments (Figure S3a). Spitfire generally had greater whole grain Fe concentrations than the other varieties.

For Zn concentrations in the aleurone layer, there was a significant interaction between variables based on a three-way ANOVA

(Variety, P treatment and AM fungi) (Figure 3b). At High P treatment, AM-inoculated grains in the Trojan variety showed significantly higher aleurone layer Zn concentrations compared to non-AM grains. In contrast, under low P treatment, AM inoculation had no effect on the aleurone layer Zn concentration across all wheat varieties (Figure 3b). These findings are further supported by the XFM scanning images of three replicate grain samples (Figure 4a). There was no significant difference in Zn absorption across the energy range in XANES in the subset of varieties (Gladius, Scepter and Spitfire), AM treatments, or the P fertiliser levels (Table S3, Figure 4b,c).

3.3 | Grain Zn and Fe bioavailability

Grain phytate content typically doubled with the addition of P (i.e., between Low to High P treatment) to the soil for all varieties. AM inoculation did not affect grain phytate levels in any variety under the Low P condition. However, under High P condition, there was a trend towards lower phytate levels in AM-inoculated plants, and this was significant in the Spitfire variety (Figure 5).

The bioavailability of Zn and Fe in grain was estimated based on the molar ratio of phytate to Zn or to Fe, respectively. Due to the

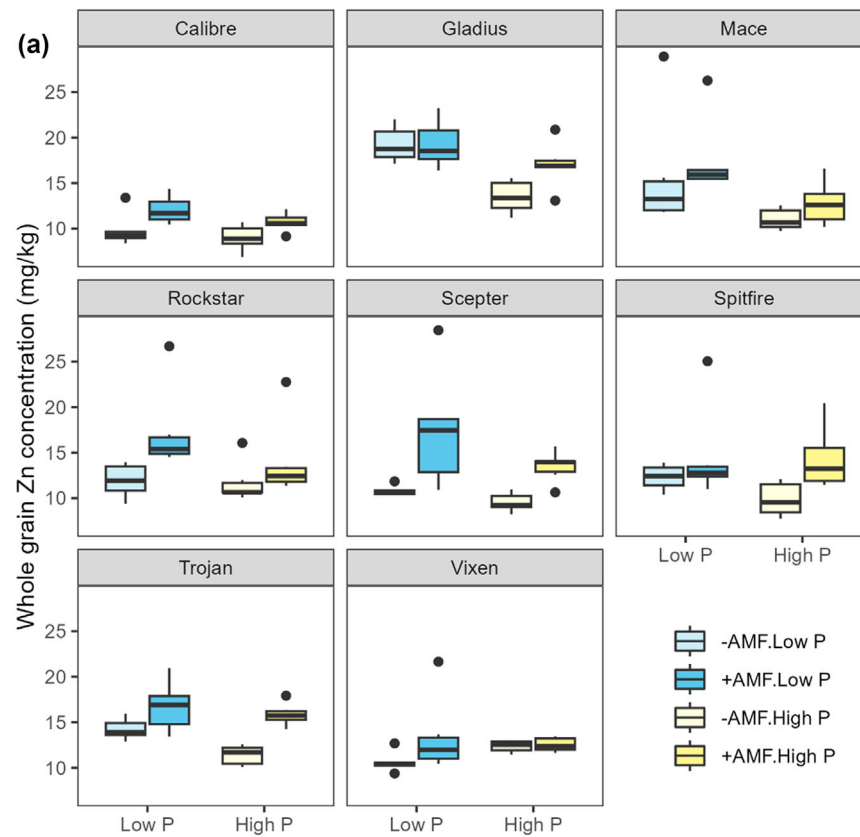
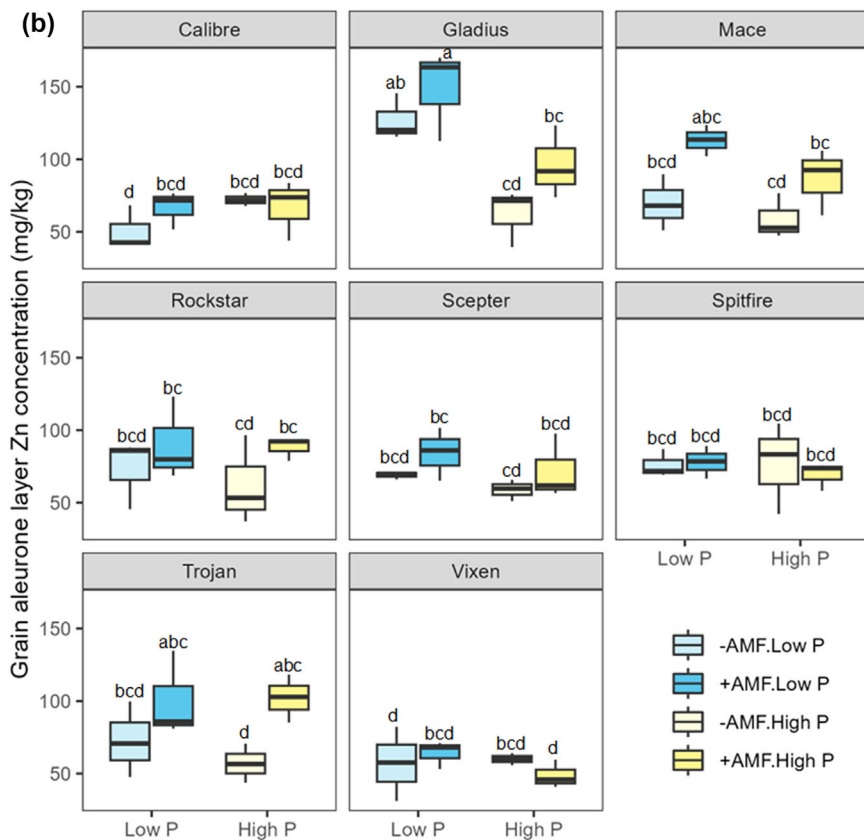


FIGURE 3 Whole grain zinc (Zn) concentration by homogenous samples Inductively Coupled Plasma (ICP) spectroscopy (a) and grain aleurone layer Zn concentration by x-ray fluorescence microscopy (XFM) (b) of eight bread wheat varieties inoculated with the arbuscular mycorrhizal fungus (AMF) *R. irregularis* (blue and yellow bars) or mock-inoculated (light-blue and light-yellow bars) and grown at low and high soil phosphorus (P) fertiliser. Thick black lines on the box-and-whisker plots are median values, $n = 6$ (ICP), $n = 3$ (XFM). Boxes that are labelled with the same letter were not significantly different at the $P < 0.05$ level (Tukey's HSD).



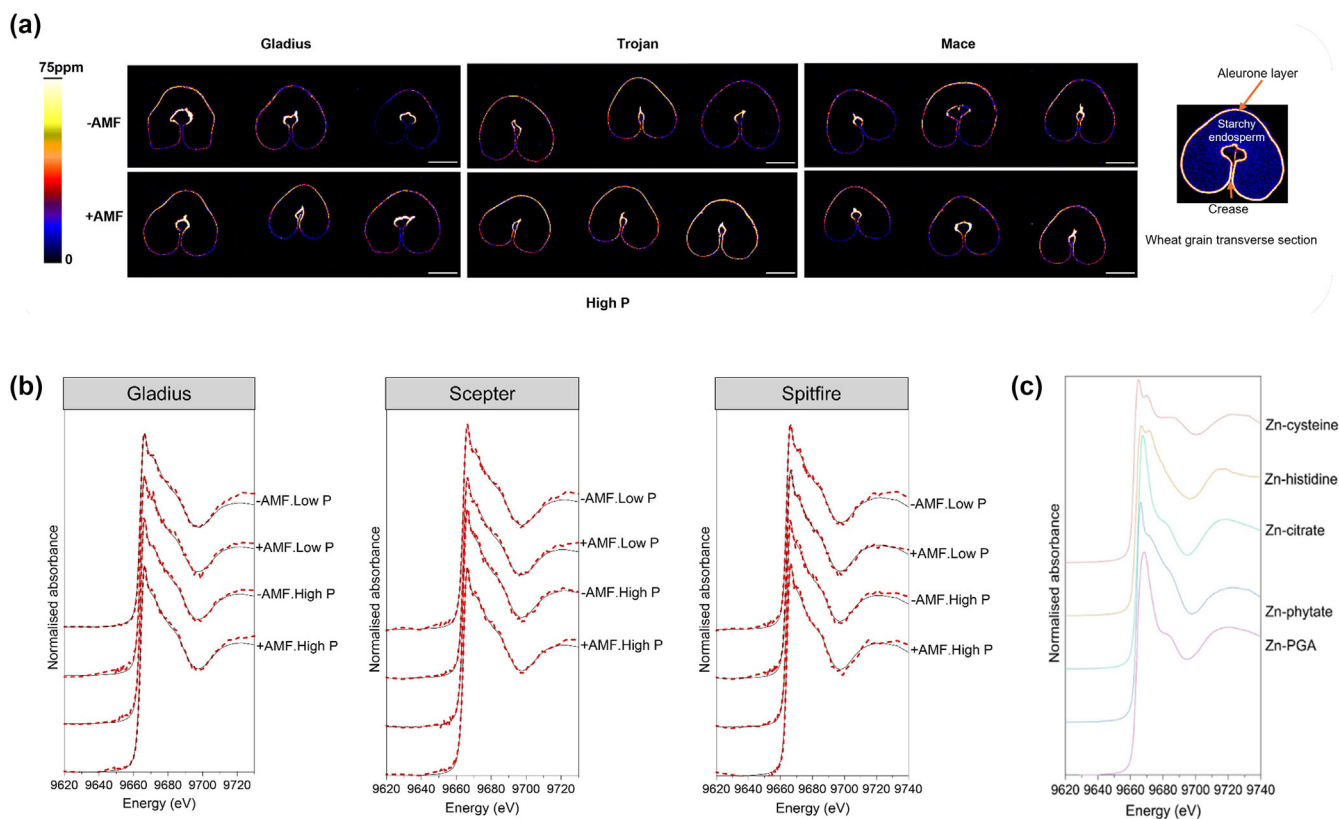


FIGURE 4 (a) X-ray fluorescence microscopy images of the distribution of zinc (Zn) in transverse grain thin sections of three bread wheat varieties (Gladius, Trojan, and Mace) inoculated with the arbuscular mycorrhizal fungus (AMF) *R. irregularis* (+AMF) or mock-inoculated (-AMF) and grown in the high phosphorus (P) soil (Colwell P of 23 mg P kg⁻¹), n = 3. The scale bar denotes 3 mm. The colour bar represents Zn concentration, ranging from low (0 ppm, black) to high (75 ppm, light yellow). Zn was more highly concentrated in the aleurone layer (blue to yellow) than in the starchy endosperm (black). (b) Normalised Zn K-edge XANES spectra (red dotted lines) of three bread wheat varieties (Gladius, Scepter, and Spitfire) inoculated with the AMF *R. irregularis* (+AMF) or mock-inoculated (-AMF) and grown in the low P and high P soil. The black solid lines show the best linear combination fit of reference spectra. XANES data were collected from a line scan across a region of interest for each grain (including the crease). The parts of the line scans corresponding to the aleurone layer (both at the periphery of the grain and in the crease) were averaged per grain to obtain Zn XANES spectra of this tissue. (c) Normalised Zn K-edge XANES spectra for the standard compounds used in the linear combination fitting (LCF).

higher Zn concentration and lower phytate concentration in AM-inoculated grain of some wheat varieties, the estimated ratio of PA:aleurone layer Zn (Zn_a) was significantly lower than the control non-AM plants, signifying greater bioavailability of Zn (Figure 6a). While the AM and non-AM plants were similar in the ratio of PA: Zn_a in the Low P treatment, in the High P treatment, the AM-inoculated plants exhibited lower ratios of PA: Zn_a . This effect was significant in Trojan and Gladius, where the PA: Zn_a ratio was reduced from 17.28 (non-AM inoculated) to 10.04 (AM-inoculated). Additionally, AM fungal inoculation reduced the molar ratio of phytate to whole grain Zn (Zn_g), especially in Gladius, Scepter and Spitfire varieties, where the PA: Zn_g ratio was reduced by approximately 50% (Figure 6b). Furthermore, the PA:whole grain Fe concentrations (Fe_g) ratio followed a similar trend as for Zn but with fewer significant differences between AM and non-AM plants; the PA: Fe_g ratio was significantly lower in the AM Calibre variety under high P treatment compared to the non-AM control plants (Figure S3b).

4 | DISCUSSION

4.1 | Mycorrhizal colonisation enhances grain biomass and nutrition of bread wheat varieties

Wheat is a pivotal crop for a large portion of the world's population, and breeding programmes are dedicated to improving traits such as yield, nutrition and resistance to biotic and abiotic stressors (Del Pozo et al., 2022; Hu et al., 2023; Langridge & Reynolds, 2021). In addition, using AM fungi as a biofertiliser in wheat production has been established as a sustainable agricultural strategy (Al-Karaki et al., 2004; Igiehon & Babalola, 2017; Sadak & Dawood, 2023). We selected eight wheat varieties that are commercially available in Australia and are cultivated for their yield performance, ranging from older varieties (Gladius, Mace, Trojan and Spitfire) to newer varieties (Scepter, Vixen, Rockstar and Calibre); their response to AM inoculation has not previously been determined.

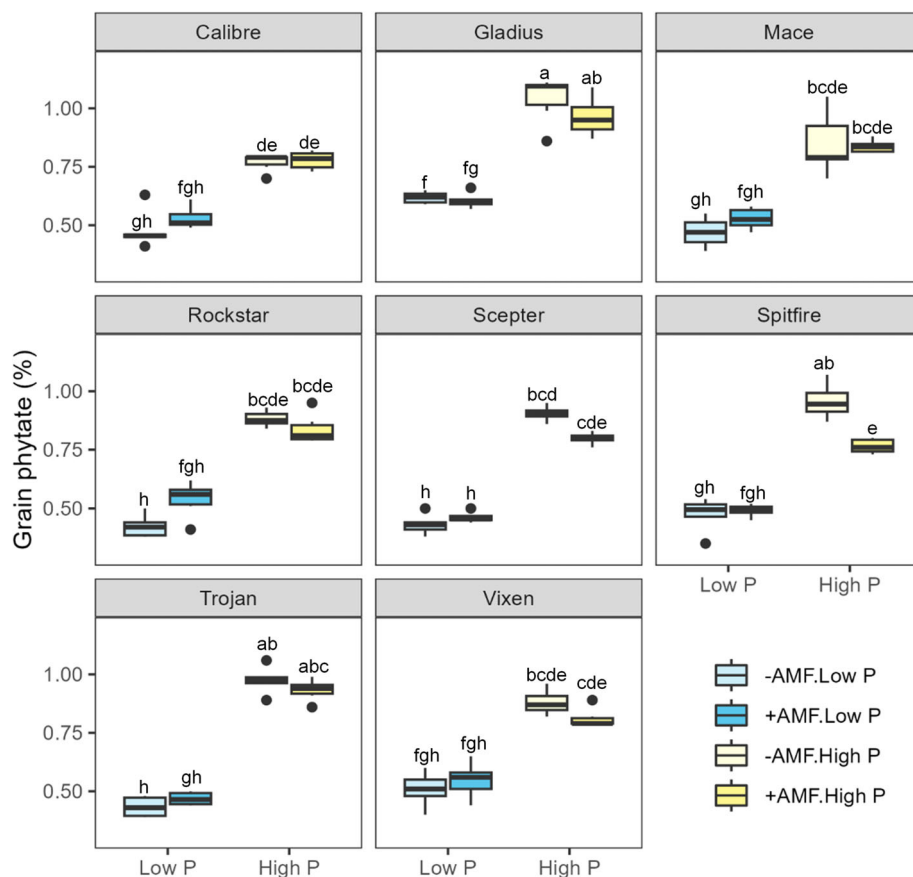


FIGURE 5 Grain phytate content of eight bread wheat varieties inoculated with the arbuscular mycorrhizal fungus (AMF) *R. irregularis* (blue and yellow bars) or mock-inoculated (light-blue and light-yellow bars) and grown at low and high soil phosphorus (P) fertiliser. Thick black lines on the box-and-whisker plots are median values, n = 6. Boxes that are labelled with the same letter are not significantly different at the P < 0.05 level (Tukey's HSD).

Colonisation by AM fungi and the response of grain yield were highly dependent on wheat variety, and they were not necessarily correlated. For example, the high AM colonisation (70%) across many wheat varieties led to significantly increased GDW in Spitfire only. The lack of correlation between AM colonisation and aboveground outcomes was also observed by Elliott et al. (2021) in a glasshouse experiment with three British wheat cultivars. Furthermore, plant responses to AM fungal colonisation can vary dramatically between and within species (Hoeksema et al., 2010; Klironomos, 2003). Responses to AM colonisation may range from increased plant growth and nutrient acquisition to neutral or negative responses where the fungal partners provide minimal or no measured benefit (Ellouze et al., 2016; Thirkell et al., 2022; Watts-Williams et al., 2019). Garcia de Leon et al. (2020) reported this from a glasshouse experiment with six spring wheat cultivars; the authors discovered that the cultivar Pikker showed the greatest positive growth response to inoculation (~20% root colonisation), while Arabella exhibited the most negative growth response (~30% root colonisation). In addition, Thirkell et al. (2022) found that plant growth response among 99 wheat lines of an Avalon × Cadenza doubled-haploid mapping population ranged from a > 30% decrease to an 80% increase in shoot biomass in response to a mixed-species AM inoculum.

The present study also demonstrated the importance of plant variety in the response to AM colonisation in terms of nutrition. While AM colonisation led to greater P uptake in many of the studied wheat

varieties, plant P uptake in Spitfire was responsive to soil P addition only. AM inoculation also had a variable effect on Zn uptake across different wheat varieties. In other work, Ercoli et al. (2017) conducted a field trial to determine the effects of AM inoculation (*R. irregularis*) on Zn and Fe uptake of two durum wheat varieties. They found that the modern variety exhibited higher grain Zn and Fe concentrations when colonised by AM fungi compared to the old variety.

4.2 | Mycorrhizal colonisation increases the bioavailability of Zn and Fe in wheat

While plant Zn and Fe uptake are important for plant nutrition, the estimated bioavailability of Zn and Fe in grain is of primary importance for human consumers of wheat products. We explored the effect of AM fungal inoculation on micronutrient bioavailability by measuring the amount of phytate, Zn and Fe in the same grain sample. Even when soil P availability was high, and phytate accumulation was expected to increase, AM fungal colonisation increased the estimated bioavailability of Zn. This is due to the dual action of (i) increased Zn concentration in the grain of AM-colonised plants and (ii) reduced accumulation of phytate in some AM-colonised plants, which act simultaneously to decrease the PA:Zn ratio in the grain. Moreover, the XFM images of grain thin sections confirmed the aleurone layer of AM-inoculated plants exhibited elevated Zn concentration compared

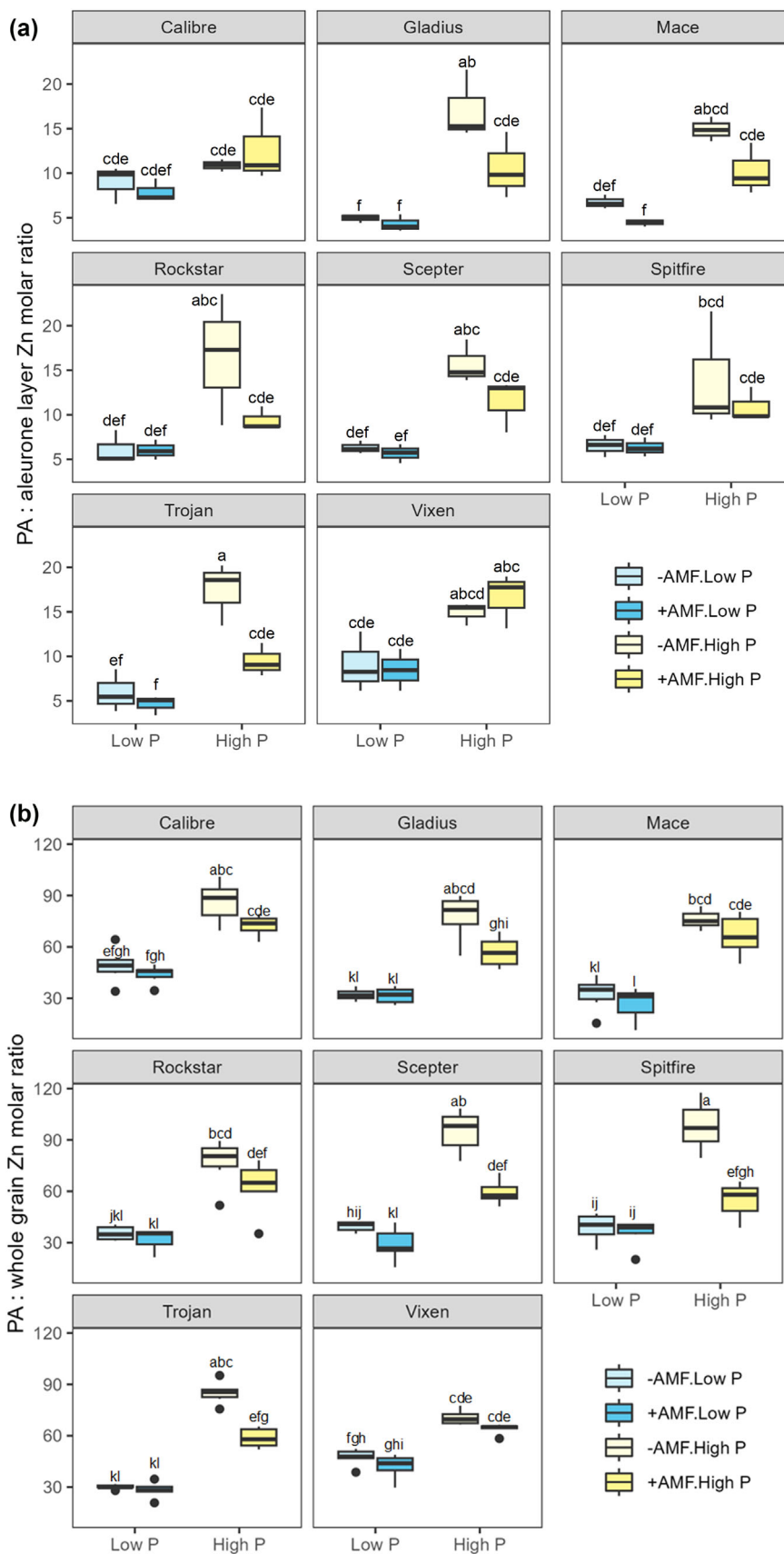


FIGURE 6 Phytic acid:aleurone layer zinc (Zn) molar ratio (a) and phytic acid:whole grain Zn molar ratio (b) of eight bread wheat varieties inoculated with the arbuscular mycorrhizal fungus (AMF) *R. irregularis* (blue and yellow bars) or mock-inoculated (light-blue and light-yellow bars) and grown at low and high soil phosphorus (P) fertiliser. Thick black lines on the box-and-whisker plots are median values, n = 6 (ICP), n = 3 (XFM). Boxes that are labelled with the same letter were not significantly different at the P < 0.05 level (Tukey's HSD).

to non-inoculated. The Zn concentration in the aleurone layer was significantly greater than that in the whole grain across all wheat varieties. The increase of aleurone layer Zn concentration in AM plants led to a substantial reduction in the molar PA:Zn_a ratio, suggesting the estimated bioavailability of Zn increased two-fold. In contrast to the results in wheat, our previous study in rice found that the stimulatory effects of AM fungi on Zn bioavailability were limited to the aleurone layer and did not extend to whole grain Zn bioavailability (Nguyen et al., 2025). The contrasting results in wheat and rice may be attributed to differences in Zn uptake pathways, Zn-binding mechanisms and also phytate accumulation via the mycorrhizal pathway in the two species due to increased P supply. While rice plants had higher phytate accumulation when colonised by AM fungi, the AM wheat plants had similar or lower phytate content than non-AM plants in both Low P and High P treatments.

The results of this study were consistent with Watts-Williams et al. (2022), who found that there was significantly higher estimated bioavailability of Zn and Fe in the AM-colonised sorghum plants due to increased Zn and Fe in the grain. Gupta et al. (2022) also reported that inoculating wheat plants with the AM fungi *R. intraradices* improved the Zn and Fe bioavailability in grains and had no effect on phytate concentration. The results from the present work suggest there is potential for AM fungi to contribute to Zn and Fe biofortification in bread wheat grain, but it is highly dependent on the variety and soil P availability.

4.3 | Soil P fertilisation increases grain biomass and phytate accumulation

Application of P fertiliser to the soil is a widespread practice in agricultural management to enhance crop yield, as soil P is the most limiting factor to yield after nitrogen and water availabilities (Bindraban et al., 2020; Rafiullah et al., 2020). The soil used in our experiment was P-limiting, as the grain biomass increased by a mean of 14% in the high P compared with the low P treatment. However, increased soil P availability or P uptake often leads directly to greater grain phytate accumulation, as was the case in the present study and others. Results from studies on 10 diverse durum wheat genotypes (Tran et al., 2021) showed that increased soil P supply consistently increased phytate in the grain, regardless of AM inoculation treatment. Furthermore, Zn and Fe concentrations may also decrease with high soil P availability due to a dilution effect (Mohammed et al., 2021; Zhang et al., 2021). In this study, the compounding effect of these two factors led to a doubling of the PA:Zn_a, PA:Zn_g and PA:Fe_g ratios when P was added to the soil, indicating a significant reduction in the bioavailability of Zn and Fe and a less nutritious flour product for human or animal consumption. This was further supported by the XANES analysis, which indicated that a greater proportion of Zn was bound to phytate in the high P compared to the low P treatments. This work suggests that in bread wheat, there is competition between maximising P reserves in the grain (desirable for plants) and achieving higher concentrations of bioavailable micronutrients

(desirable for humans). However, selecting or generating wheat genotypes that minimise the plant's innate phytate accumulation, in conjunction with AM inoculation, may lead to higher micronutrient bioavailability without compromising agronomic practices or grain yield.

5 | CONCLUSION AND FUTURE WORK

Our research indicates that AM inoculation can have positive effects on the nutritional quality of bread wheat. AM-inoculated plants exhibited greater Zn concentrations compared to non-inoculated, and there were distinct patterns observed between Zn concentrations in whole grains compared to in the aleurone layer. We also found that AM inoculation did not affect grain phytate content under low soil P conditions and even reduced phytate levels in some varieties under high soil P, leading to greater estimated bioavailability of Zn and Fe in grains in the AM plants. This suggests there is no trade-off between grain yield and nutritional quality of AM plants when P fertiliser is applied.

AUTHOR CONTRIBUTIONS

TDN was involved in the design of the experiment, performed the research and data analysis, contributed to data interpretation and wrote the manuscript as it appears in its final form. AATJ contributed to the data interpretation and writing of the manuscript. EL, ES and CD were involved in the design of the experiment and performed the research, data analysis and writing of the manuscript. SJWW was involved in the design of the experiment and contributed to data analysis, interpretation and writing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request to the corresponding author.

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SUPPORTING INFORMATION

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