Bioavailability of zinc and iron in durum wheat: A trade-off between grain weight and nutrition?

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Summary:
• The bioavailability of micronutrients (zinc [Zn] and iron [Fe]) in cereal crops such as durum wheat is critically important for human nutrition. Bioavailability is a product of complex interactions between plant phosphorus (P) uptake and storage in grain (as phytate), and plant micronutrient uptake. The bioavailability of Zn and Fe in cereal grain is affected by soil nutrient concentrations and associations with arbuscular mycorrhizal (AM) fungi, but has been scarcely studied.

• A geographically diverse collection of 101 durum wheat genotypes was surveyed for grain bioavailability of Zn and Fe. Ten genotypes were then selected and grown with and without AM fungal inoculation and soil P fertilization to understand the effects of manipulating soil P availability and uptake on micronutrient bioavailability.

• The strongest negative effect on grain micronutrient bioavailability was soil P fertilization, however, it also led to increased grain weight. Crop variety selection had the greatest variation in the P-fertilized soil, but AM fungal inoculation had a positive effect on bioavailability in one variety in the non-fertilized soil.

• In order to grow more nutritious durum wheat crops, variety selection and AM fungal inoculation are important considerations. In general, there is a trade-off between grain weight (yield) and micronutrient bioavailability in grain that could be addressed through breeding P-deficiency tolerant varieties.

Societal Impact Statement
Micronutrients such as zinc and iron are critical for human health. For the world’s population that relies on cereal products to obtain micronutrients, the bioavailability (absorption of nutrients in the gut) can be hindered by an anti-nutritional compound, phytate. Phytate accumulation in grain is affected by soil properties including phosphorus availability and arbuscular mycorrhizal (AM) fungi. Here, we investigated the effects of AM fungi and soil phosphorus fertilization on micronutrient bioavailability in durum wheat and found that fertilization greatly decreased the bioavailability of micronutrients, but AM fungi can take up more micronutrients, which can lead to improved bioavailability when the soil is not fertilized.
**INTRODUCTION**

Staple cereal crops are important providers of calories and mineral nutrients to the world’s population, but reliance on cereal-based diets can lead to micronutrient malnutrition and poor human health (White & Broadway, 2009). It is estimated that more than 30% of the world’s population suffer from “hidden hunger” as a result of deficiency in their iron (Fe), zinc (Zn), vitamin A, and/or iodine dietary intake (FAO, 2013). Thus, not only is there a need to increase global food production but also the production of food with greater nutritional quality. The inclusion of nutritional traits (e.g., Zn and Fe, and their bioavailability) is, therefore, an important consideration for breeding programs (Cakmak, 2008; Cakmak, Pfeiffer, & McClafferty, 2010).

Many studies have measured the concentration of Zn and Fe in grain and, importantly, extended this to estimating or measuring their bioavailability (ability to be absorbed in the digestive tract) (Beasley et al., 2019; Welch, House, Ortiz-Monasterio, & Cheng, 2005; Zhang, Liu, Liu, Chen, & Zou, 2017). Bioavailability of Zn and Fe in grain depends heavily on the concentration of phytic acid (PA)—the dominant storage form of phosphorus (P) in seeds. PA in grain can strongly chelate nutritionally important minerals including Zn and Fe, making them unavailable when consumed by humans (Reddy, 2001). Therefore, PA is considered a primary anti-nutritional compound in grain which may contribute to human Zn and Fe malnutrition (Welch & Graham, 2004).

Arbuscular mycorrhizal fungi are the association between arbuscular mycorrhizal (AM) fungi and the roots of many important agricultural plant species including wheat, barley, sorghum, and many horticultural crop species including varieties of tomato and bean (Tran, Watts-Williams, & Cavagnaro, 2019; Zhang, Lehmann, Zheng, You, & Rillig, 2019). Colonization by AM fungi can improve cereal crop yield and nutrition, and importantly can increase the uptake of Zn and Fe into the grain of cereal crops (Ercoli, Schübler, Arduini, & Pellegrino, 2017; Lehmann & Rillig, 2015; Lehmann, Veresoglou, Leifheit, & Rillig, 2014; Watts-Williams & Gilbert, 2020), and the edible parts of other food crops (e.g., legumes, tomato fruit) (Cavagnaro et al., 2006; Pellegrino & Bedini, 2014). Due to their capacity to improve crop nutrition and capacity to confer other benefits such as drought, salinity, and weed tolerance, as well as soil ecosystem services, AM fungi may be part of the solution to improve food security in a changing climate (Antunes et al., 2012; Rillig et al., 2019; Thirkell, Chaters, Elliott, Sait, & Field, 2017; Wipf, Krajinski, van Tuinen, Recorbet, & Courty, 2019).

One challenge of using AM associations for food security is that the effect of AM fungal colonization on plant growth and nutrition is highly variable and dependent on many factors. The list of interacting factors is lengthy (see Pickles, Truong, Watts-Williams, & Bueno, 2020 for a comprehensive list), but the main drivers include: plant species and variety choice, soil physical characteristics, including nutrient availability and biological characteristics (microbiome), and colonizing AM fungal species, abundance, and diversity. The variation in plant growth response to AM fungal colonization in diverse plant genotypes has been illustrated in many staple food crops such as bread wheat (Zhu, Smith, Barritt, & Smith, 2001), durum wheat (Elliouze et al., 2015), and sorghum (Watts-Williams et al., 2019). There is also demonstrated diversity in the effects of AM fungi on cereal nutrient uptake and consequently nutritional quality of their food parts, for example, in barley (Al Mutairi et al., 2020) and sorghum (Cobb et al., 2016), and water relations in tomato (Bowles, Barrios-Masias, Carlisle, Cavagnaro, & Jackson, 2016) and *Medicago truncatula* (Watts-Williams, Cavagnaro, Tyerman, 2019). For the purpose of harnessing the potential for AM fungi to contribute to sustainable agriculture, the selection of major crops should, therefore, be based on the selection of the genotypes that are compatible with AM fungi (Rillig et al., 2019; Singh, Hamel, DePauw, & Knox, 2012).

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is one of the most important commercial food crops, and is grown globally. Durum wheat semolina is used for pasta production and other specialty foods that are consumed as staple food across the world (Matsuo, 1994). Thus, quantifying the variation in bioavailability of essential micronutrients such as Zn and Fe in diverse durum wheat genotypes is critical for enhancing human nutrition (Magallanes-López et al., 2017). Although the growth response of durum wheat to AM colonization is generally relatively low (Al-Karaki, 1998; Tran, Watts-Williams, et al., 2019), the benefit of AM fungi to durum wheat can be observed under certain conditions and when investigating specific variables such as bioavailability in grain (Ercoli et al., 2017; Tran, Cavagnaro, & Watts-Williams, 2019). Here, we have built on our recent finding that inoculation with AM fungi increased PA concentration and, thus, reduced the estimated bioavailability of Zn and Fe in a single variety of durum wheat. We had three specific research questions that centre around three agronomic management practices: (i) crop genotype selection, (ii) soil P fertilization, and (iii) AM fungal inoculation:

(i) How do the diverse genotypes of durum wheat vary in terms of grain weight per plant, nutrient, and phytate concentrations?

(ii) To what extent does soil phosphorus fertilization affect the grain weight per plant and nutrition (including bioavailability) of durum wheat?

(iii) To what extent does inoculation with AM fungi affect durum wheat nutrition and micronutrient bioavailability?

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**KEYWORDS**

Arbuscular mycorrhizal fungi, durum wheat, iron, micronutrient bioavailability, phytate, zinc
2 | MATERIALS AND METHODS

2.1 | Experiment 1

A panel of 101 geographically and genetically diverse durum wheat genotypes (listed in Table S1) was used to estimate the diversity in grain phytic acid accumulation. This set was chosen from a core population of 315 genotypes (see Liu, Bruce, Sissons, Able, and Able (2018)). The seeds of 100 genotypes (DBA-Aurora excluded) were harvested in 2014 from a field trial in Breeza, New South Wales, Australia (31.25°S, 150.46°E). The seed of commercial variety DBA-Aurora was sourced from two different field seasons (2016 and 2017) for the experiments conducted (Roseworthy, South Australia, 34.54°S, 138.69°E). All seed samples (approximately 5 g of each genotype) were dried in an oven at 60°C for 72 hr, then ground to a fine powder to homogenize using a Retsch mill MM400 (Germany). Two weighed subsamples of whole-grain flour were taken to analyze the phytic acid (PA) and the Zn and Fe concentrations in the seed. The bioavailability of Zn and Fe, including the molar ratio of PA to Zn and of PA to Fe, was calculated as is the standard for estimating the bioavailability of Zn and Fe, respectively (Abizari et al., 2012; Morris & Ellis, 1989). Based on the results from 101 genotypes, a subset of 10 genotypes were then chosen for further study based on their commercial importance and geographic origin; the genotypes with the lowest (Om Rabi 6) and highest (Chapala 67) PA concentrations were selected, as well as four Australian and four Italian varieties with contrasting PA concentrations.

2.2 | Experiment 2

2.2.1 | Soil and plant preparation

The soil used was a 9:1 (w/w) mixture of sand and field soil. This field soil was collected from the grounds of the University of Adelaide’s Waite Arboretum, Australia (~34.969209, 138.631397). The field soil was sieved to <2 mm to eliminate any coarse debris, autoclaved twice, and oven dried at 60°C before being mixed thoroughly with the washed and autoclaved fine sand and is referred to as “soil” hereafter. The field soil was a loam classified as an Urrbrae red-brown earth (Alfisol) with 6.14 pH, 55 mg N/kg KCl-extractable ammonium, 11 mg N/kg KCl-extractable nitrate, 13.5 mg P kg⁻¹ of Colwell P, 81 mg DTPA-extractable Fe kg⁻¹, and 17 mg DTPA-extractable Zn kg⁻¹. The mixing resulted in the soil having 19 mg DTPA-extractable Fe kg⁻¹. The AM fungal inoculum used contained dry soil, spores, external hyphae of *Rhizophagus irregularis* WFVAM10, and root fragments of previously colonized Marigold (*Tagetes patula*) plants. In the AM fungal treatment pots, 140 g (10% pot weight) of the *R. irregularis* WFVAM10 inoculum (synonymous with DAOM 181,602, an earlier voucher number for DAOM 197,198, formerly named *Glomus intraradices*) was mixed thoroughly with 1,260 g soil. Meanwhile, the other half of the pots were mock inoculated whereby each pot of 1,400 g soil was administered with 15 ml of an aqueous filtrate made from a 20% suspension (w/v) of the *R. irregularis* inoculum, filtered twice using Whatman #1 paper (Li, Smith Sally, Holloway Robert, Zhu, & Smith, 2006), and mixed well through the soil.

To investigate the effects of soil P availability on durum wheat, half of the pots were amended with 20 mg P kg⁻¹ soil in the form of KH₂PO₄ solution to both AM fungal- and mock-inoculated pots. This created two soil P availability treatments, without and with P addition with Colwell P concentrations of 7.8 and 25.2 mg P kg⁻¹, respectively. The two P treatments are referred to as Low P and High P hereafter. Seeds of the 10 selected durum wheat genotypes were sterilized in 10% sodium hypochlorite solution for 5 min, then rinsed with running reverse osmosis (RO) water before being germinated at 25°C in the dark for 2 days. Germinated seeds were transferred to autoclaved sand and grown on a greenhouse bench for 10 days. The seedlings were then transplanted to the previously prepared pots, with one plant per pot. Each treatment was replicated five times, to a total of 200 pots.

2.3 | Plant growth and harvest

The durum wheat plants were grown in a controlled environment greenhouse on the Waite Campus of The University of Adelaide, during July to October 2019. Temperature in the greenhouse ranged from 10.8 to 29.1°C; there was supplemental overhead lighting that provided a 16/8 hr day/night photoperiod throughout the growing period. The pots were arranged in a randomized design on the greenhouse benches and were re-randomized once per week. Plants were watered to 10% of the soil weight, three times weekly with RO water. Once per week, the pots were fertilized with 10 ml of modified Long-Ashton solution without P (Cavagnaro, Dickson, & Smith, 2010). Plants were also amended with 30 mg N per pots in form of NH₄NO₃ solution at the second, fourth, and sixth week of the growing course to a total of 90 mg N per pot by harvest.

All plants were destructively harvested 77 days after planting. The durum wheat spikelets were cut from the rest of the aboveground biomass, which was then cut at soil level, and roots were washed free of the soil. The fresh shoot and root weight of each plant were then determined. Between 100 and 300 mg of fresh roots were subsampled and placed into a 50% ethanol solution. After drying at 60°C for 72 hr, the shoot biomass of each plant was weighed. The grain from each plant was then separated using athreshing board; then, the grain dry weights were determined per plant. Grain samples were then ground to fine whole-grain flour as described previously, and two subsamples were taken for determination of: PA concentration and nutrient concentrations.

2.4 | Sample analysis

Grain PA concentration was measured using a phytic acid/total phosphorus assay kit (Megazyme, Ireland) to measure PA concentration in
durum wheat grain following the manufacturer’s protocol. To estimate the bioavailability of Zn and Fe in cereal grain, the molar concentrations of PA, Zn, and Fe were, respectively, calculated, and the ratio of PA to Zn or Fe was used as a predictor of bioavailability (Reddy, 2001). In the case of Zn, PA:Zn ratios below 5, between 5 and 15, or >15 correspond to high, medium, and low bioavailability of Zn in a grain sample (Gibson, 2006). Likewise, a grain sample with a PA:Fe ratio >1 was considered to have low Fe bioavailability (Hurrell & Egli, 2010).

Grain elemental concentrations were determined as follows: 200 mg of finely ground whole-grain flour was cold digested overnight and then hot digested in a mix of 2 ml nitric acid and 0.5 ml hydrogen peroxide (Miller, 1998). The acid digests were then diluted and analyzed for concentrations of Zn, Fe, and P by inductively coupled plasma atomic emission spectroscopy ICP-AES (Thermo Jarrell Ash Corp.).

Arbuscular mycorrhizal colonization of roots was determined as follows: fresh roots fixed in 70% ethanol for >48 hr were rinsed with RO water and then cleared in a 10% (w/v) potassium hydroxide solution at room temperature for 7 days. Cleared roots were then rinsed and stained in 5% ink in vinegar solution (modified from Vierheilig, Coughlan, Wyss, and Piche (1998)) at 60°C for 10 min before being de-stained in acidified water for 12 hr, then washed and moved to 50% glycerol solution for storage. Arbuscular mycorrhizal colonization of roots was estimated by microscope on at least 100 intersects using the gridline intersect method (Giovannetti & Mosse, 1980).

### 2.5 Statistical analysis

A mixed effects model was employed to analyze the data using the “lme” function within the “nlme” package in R version 4.0.2 (R Core Team, 2019). The mixed effects model included Mycorrhiza and Phosphorus as fixed factors and Genotype as a random factor. Due to the strong influence of Phosphorus treatment (Low and High P) on every response variable measured, the model included an additional correction for Phosphorus using the varIdent() function within the “nlme” package. For AM colonization, only the AM fungi-inoculated plant data were included, and the Mycorrhiza term removed from the model. Where the interaction between Mycorrhiza and Phosphorus was significant (p < .05), the “lsmeans” package and function were used to conduct pairwise comparisons between the treatments and identify any significant differences.

In order to identify effects of AM inoculation in individual durum wheat varieties, targeted Student’s t tests were conducted for each Phosphorus and Genotype treatment, respectively, for each response variable, using the “rstatix” package. Any significant differences between the AM and non-AM means are denoted on the respective Figure.

A principal components analysis (PCA) was undertaken for Low P and High P separately using the “PCA” function in the “FactoMineR” package, including all of the grain mass and nutrition data. Prior to analysis, the data were standardized to the same scale using “scale. unit = TRUE” within the “PCA” function. The PCA biplots were drawn using the “factoextra” package and the loadings colored by levels of Mycorrhiza (either AMF or Mock); the group mean was also computed for each level, and a 95% confidence ellipse drawn around the mean to determine significant differences between groups.

### 3 RESULTS

In the first experiment (Experiment 1, hereafter), the concentration of PA and estimated bioavailability of Zn and Fe of 101 geographically diverse varieties of durum wheat were analyzed. This informed our choice of 10 genotypes that were used in Experiment 2 to examine the responses to AM fungi under low and high soil phosphorus conditions, in terms of grain weight per plant and nutrition (including Zn and Fe bioavailability).

#### 3.1 Experiment 1

**3.1.1 The bioavailability of Zn and Fe in grain from 101 durum wheat genotypes**

Nutritional traits including PA, Zn, and Fe concentrations were measured on grain from 101 genetically diverse durum wheat genotypes grown under standardized field conditions and ground to a whole-grain product. The concentration of PA in the diverse genotypes ranged from 0.62% in Om Rabi 6 (Syria) to 1.26% in Chapala 67 (Mexico) (Figure 1). In addition, there was a weak positive correlation (R² = 0.283; p < .0001) between the PA and P concentrations in grain across the 101 genotypes. The molar ratios of PA:Zn in the 101 durum wheat genotypes ranged from 36.6 to 101.4 (Figure S1a), and the molar ratio of PA:Fe ranged from 8.3 to 25.2 (Figure S1b).

**3.2 Experiment 2**

**3.2.1 The effect of soil P availability on response variables**

The addition of P fertilizer to the soil (High P) increased grain weight, and the concentrations of P and PA in the grain, but it also decreased concentrations of grain Zn and Fe, compared to the Low P treatment (Table 1). The combination of increased PA alongside decreased Zn and Fe in the grain meant that PA:Zn and PA:Fe ratios increased markedly in the High P treatment, by three and five times, respectively. Thus, the estimated bioavailability of Zn and Fe in High P grain was heavily decreased compared to in the Low P grain.

#### 3.3 Grain weight and AM colonization

The Italian genotype Iride had the greatest grain weight per plant of the 10 genotypes in both soil P treatments, but especially when not inoculated with AM fungi. At Low P, Iride’s mean grain weight
the concentration of the important micronutrients Zn and Fe was measured in the grain samples in order to assess how different durum wheat genotypes manage uptake from the soil, and the effects of AM colonization.

Both grain Zn and Fe concentrations were highly influenced by AM fungal inoculation and soil P availability, and this was reflected in the interaction between Phosphorus and Mycorrhiza. For both grain Zn and Fe, the concentrations were higher in the AM plants at Low P, than the other three treatments. At Low P, the Zn concentration was highest in Chapala 67 (209.04 mg Zn kg\(^{-1}\)) while Iride was the lowest (86.51 mg Zn kg\(^{-1}\)) (Figure S3). At High P, Chapala 67 maintained the highest grain Zn concentration (150.70 mg Zn kg\(^{-1}\)), while Iride again had the lowest concentration (61.74 mg Zn kg\(^{-1}\)). In the case of grain Fe concentration, Tjilkuri was the highest (91.85 mg Fe kg\(^{-1}\)) and Om Rabi 6 the lowest (51.7 mg Fe kg\(^{-1}\)) (Figure S4). At High P, the highest grain Fe concentration was observed in Chapala 67 (69.68 mg Fe kg\(^{-1}\)), while Iride was the lowest (44.11 mg Fe kg\(^{-1}\)).

### 3.5 Grain phytate concentrations

For grain PA concentration, at Low P, the grain from Chapala 67 had the greatest PA concentration (mean 0.94%) and the...
Australian genotype Tjilkuri had the lowest grain PA concentration (mean 0.39%) (Figure 3). But at High P, Australian genotype Duramba had the highest grain PA (mean 1.45%), while Iride had the lowest (mean 1.35%) (Figure 3b). When targeted t tests were conducted on the Low P data to tease out effects of AM fungal inoculation, Simeto and Duramba had higher grain PA concentrations when inoculated with AM fungi than when mock inoculated.

Grain P concentration (Figure S5) displayed a strong relationship with PA concentration ($R^2 = 0.82, p < .0001$) when all data were considered. However, when the Low P and High P data were split, the relationship between P concentration and PA was much stronger at Low soil P availability ($R^2 = 0.71, p < .0001$) than at High P ($R^2 = 0.33, p < .0001$). This suggests that: (a) grain P concentration could be used as a reliable estimate for PA concentration only when soil P is limiting to the plant, and (b) when P is not limiting, diverse durum wheat genotypes do not consistently allocate the same amount of grain P to phytate.

3.6 | Grain Zn and Fe bioavailability

The calculation to estimate Zn and Fe bioavailability in the grain is a product of the amount of PA and the amounts of Zn and Fe measured on the same sample. As AM inoculation is known to affect both PA and Zn and Fe uptake in cereal crops, the resulting PA:Zn and PA:Fe ratios provide a simplification of complex below-ground interactions.

The molar ratio of PA to Zn estimates Zn bioavailability, and there was considerably more variation in this ratio when plants were grown at High P than at Low P (Figure 4). At both Low and High P, Iride had the greatest PA:Zn ratio (5.9 at Low P and 18.2 at High P). The lowest PA:Zn ratios were found in DBA-Aurora (mean 4.3) when grown at Low P, and in Chapala 67 (mean 9.2) when grown at High P. Targeted t tests revealed that the PA:Zn ratio was higher in the non-AM Iride plants at Low P.

The estimate of Fe bioavailability in the grain samples (PA:Fe) showed more variation at Low P than at High P, contrary to the
results for PA:Zn (Figure 5). Chapala 67 had the greatest PA:Fe ratio (mean 9.6) and Tjilkuri had the lowest grain PA:Fe ratio (mean 4.5). At High P, Iride had the highest mean PA:Fe ratio (21.3) and Chapala 67 had the lowest ratio of 16.7 (Figure 5b). Only Iride had a greater PA:Fe ratio in the AM plants than the non-AM plants, at Low P (targeted t test).

3.7 | Principal components analysis

A PCA biplot was constructed for the 10 durum wheat genotypes; the analysis was split between Low P and High P and included measures of grain weight, concentrations of P, Zn, Fe, Mn, Cu, and phytate, and ratios of PA:Zn and PA:Fe.

For the Low P data, principal component (PC) 1 accounted for 54.7% of the variability in the data and was driven by concentrations of P, Zn, Mn, and phytate in the positive direction, and grain dry weight in the negative direction (Figure 6a). PC2 accounted for a further 23.9% of the variability in the data and was driven by PA:Zn and PA:Fe ratios (positive). The confidence ellipses on the biplot depict separation of the mean AM treatment from the mean non-AM treatment (Figure S6a). There was also some separation between durum wheat genotypes based on their grain characteristics; for example, Chapala 67 was significantly different from all the other genotypes except Jandaroi, owing to its high phytate, P, and Zn concentrations.

At High P, PC1 accounted for more variation than at Low P (72.2%), and was driven by all of the grain nutrient concentrations in one direction (positive), and by grain weight and Zn and Fe bioavailability in the other direction (negative) (Figure 6b). PC2 accounted for just an additional 15.6% of the variability in the data, and was driven primarily by phytate concentration in the grain. In this case there was no significant separation between Mycorrhiza treatments (indicated by confidence ellipses overlapping on the
biplot; Figure S6b). Separation of the durum wheat genotypes primarily occurred along PC1 in this instance; Iride and Chapala 67 were the most distant genotypes, with Iride clustering toward higher grain weight and lower micronutrient bioavailability, and Chapala 67 toward greater nutrient concentration in grain. The three other Italian varieties clustered quite tightly together along with Om Rabi 6 and DBA-Aurora.

4 | DISCUSSION

This study focused on the effects of three components of agronomic management on durum wheat grain yield micronutrient bioavailability: (a) crop genotype selection, (b) soil P fertilization, and (c) AM fungal inoculation. The results are now discussed in the context of improving agronomic management for greater grain micronutrient bioavailability and, ultimately, human nutrition.

4.1 | Plant genetic diversity greatly influences the bioavailability of essential micronutrients

Durum wheat is an important food crop for much of the world’s population; numerous breeding programs exist to improve desirable traits of the crop for flour and pasta production, including yield, protein, resistance to pathogens, etc. (Alahmad et al., 2018; Vita & Taranto, 2019). By comparison, Zn and Fe concentrations, and bioavailability, in durum wheat has been afforded less attention and effort as breeding targets (Cakmak et al., 2010). However, there are other crops such as bread wheat and rice that have had considerable improvements made to their Zn and/or Fe bioavailability for human nutrition, both through conventional breeding and genetic engineering (Stangoulis, Huynh, Welch, Choi, & Graham, 2007). Breeding for improved bioavailability of Zn and Fe in cereals may be a challenging task, as bioavailability is the product of complex interactions between grain size, phytic acid accumulation (P storage), and Zn and Fe concentrations.

FIGURE 4 Molar ratios of phytic acid to Zn of 10 durum wheat varieties either inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (pink) or mock inoculated (blue) and grown at Low or High P. Values are mean ± standard error of the mean, n = 5. See Table 1 for outcomes of the linear mixed effects model.
Fe uptake. This makes it difficult to pinpoint just one or two genetic markers or targets for improved Zn/Fe bioavailability—an issue for many breeding programs (Gilliham, Able, & Roy, 2017). All of these factors are, in addition, affected by colonization of the plants by AM fungi. Working on one target, for example, increasing Zn loading into grain endosperm (Menguer et al., 2018; Schroeder et al., 2013), or reducing PA accumulation in grain (Raboy, 2001, 2009), at least brings in key knowledge that can ideally eventually be combined.

A promising finding from this study is the marked variation in the amount of grain PA when diverse genotypes are grown under standardized environmental conditions. When a set of 101 geographically diverse durum wheat genotypes were analyzed, we found a twofold variation in PA concentration. This variation is similar to the finding of Magallanes-López et al. (2017), who used the same method described here for PA determination, and reported a two-fold difference in PA concentration between the lowest (0.46%) and highest (0.95%) genotypes across 46 geographically diverse durum wheat. Ficco et al. (2009) used a rapid assay for the indirect determination of grain PA, and found only a 1.65-fold variation in estimated PA concentration (0.46–0.76 mg P\textsubscript{i} g\textsuperscript{-1}) across 84 Italian durum wheat cultivars. In general, the PA concentrations were lower in Ficco et al. (2009) and Magallanes-López et al. (2017) than in our study; and this is likely due to differences in the field/growing conditions, for example, higher soil P availability in our study may have contributed to the greater PA accumulation. Indeed, Ficco et al. (2009) demonstrated that environmental conditions (three different field sites) led to differences in grain PA concentration in the same set of cultivars, and the samples with the highest grain PA concentration were grown in the soil with the highest P availability.

In order to begin addressing the complexity of micronutrient bioavailability across cereal genetic variation, Zn and Fe concentration was measured on the same samples, allowing for calculation of molar ratios of PA to Zn and Fe. These molar ratios provide an estimation of Zn and Fe bioavailability for human nutrition. Because...
Zn/Fe accumulation in cereal grain is highly variable, and is independent of PA accumulation, it is not surprising that the variation in these ratios was even higher than that of PA concentration alone. The range of PA:Zn ratios had a 2.85-fold variation (35.6–101.4) and the PA:Fe was similar, with 3-fold variation (8.3–25.2). The variation found here in the set of 101 durum wheat genotypes was greater than that found in 46 genotypes previously, where the PA:Zn ratio displayed variation of 1.4-fold (16.9–23.6) and PA:Fe of 2.45-fold (12.1–29.6) (Magallanes-López et al., 2017). Although molar ratios only provide an estimate of bioavailability, it is important to note that all of the genotypes used in our study and that of Magallanes-López et al. (2017) fell into the “low” bioavailability category for both Zn and Fe, according to the published standards (Gibson, 2006; Hurrell & Egli, 2010). This highlights that for durum wheat, the current selection of genetic material for other desirable traits has not overcome the complex challenge of improving both Fe and Zn concentrations in grain while reducing PA concentration; however, the potential benefits of doing so could be highly impactful on human health (Cakmak, McLaughlin, & White, 2017).

### 4.2 Soil P fertilization improves grain weight per plant but reduces micronutrient bioavailability in grain

In the context of agricultural management practice, P fertilizer is applied to the soil to amend plant-available P deficiency and consequently increase crop yield; this was the outcome for the durum wheat plants here, with a mean increase of 78% in grain weight per plant when P was applied. However, increased soil P availability also led to reduced bioavailability of Zn and Fe in two keys ways: reduced concentrations of Zn and Fe in the grain (a product of increased grain yield), and increased PA concentrations. The compounding effect of these two factors meant that the ratios of PA to Zn and Fe both increased by three times, which greatly reduces bioavailability for consumers of the flour product. So, there appears to be a trade-off between achieving greater grain weight per plant, or a grain product that is more highly concentrated in micronutrients that are also more bioavailable. This hypothesis for trade-off is supported by the PCA biplot that shows grain dry weight and higher PA:Zn/PA:Fe ratios (lower bioavailability) all driven in the negative direction, in the High P data only.

In the case of bread wheat, rice, and other cereal crops, efforts have been made to breed varieties that are more efficient at P acquisition, and therefore can thrive under low soil P conditions (Campos et al., 2018; Ramaekers, Remans, Rao, Blair, & Vanderleyden, 2010; Vandamme, Rose, Saito, Jeong, & Wissuwa, 2016). Such varieties may offer an option where that trade-off is not necessary; that is, varieties where both yield and micronutrient bioavailability are optimized because grain PA concentration is kept low. However, this relies on the varieties being grown without substantial amounts of fertilizer, and there would still be a yield penalty compared to a P-fertilized soil. But this may be a more attractive option where agricultural soils are naturally low in P such as many of the grain growing regions of Australia (Kooyman, Laffan, & Westoby, 2017), or due...
to the rising cost of inputs such as inorganic fertilizers (Brunelle, Dumas, Souty, Dorin, & Nauda, 2015; Mew, 2016). Even within the 10 durum wheat genotypes grown in this study there was evidence for such a compromise; the Australian varieties Tjilkuri and DBA-Aurora achieved modest grain yield in the P-deficient soil compared to the other varieties, but also had relatively low accumulation of PA in their grain (Tjilkuri was the lowest). This led to DBA-Aurora and Tjilkuri having the lowest PA:Zn and PA:Fe ratios of the 10 genotypes, and in the case of Zn, the ratios for each were within the “high” bioavailability category (<5). These two Australian varieties have been bred for wide adaptation across Australia’s grain growing regions, which may help explain their resilience (in terms of grain yield) to the P-deficient soil in this study.

4.3 | Arbuscular mycorrhizal fungi interact with plant P, Zn, and Fe uptake to affect grain phytate

In general, AM fungi can increase the grain concentration of P, but also of Zn and Fe in durum and bread wheat varieties (Ercoli et al., 2017; Lehmann & Rillig, 2015; Lehmann et al., 2014). In terms of grain yield, wheat is generally non-responsive to AM inoculation (i.e., there is no increase or decrease in yield compared to a control) (Ellouze et al., 2015). This makes AM fungal inoculation challenging to manage in terms of enhancing grain micronutrient bioavailability, as it directly or indirectly affects all the plant factors that combine to determine bioavailability.

In a previous study using the same AM fungus, we found that the AM durum wheat (DBA-Aurora) plants had higher grain concentrations of PA; consequently, the predicted bioavailability of both Zn and Fe was lower in the AM than in the non-AM plants (Tran, Cavagnaro, et al., 2019). Of the 10 genotypes analyzed here, inoculation with AM fungi led to increased grain PA concentration only in Simeto and Duramba, and for the rest there was no significant effect. In contrast to the results from Tran, Cavagnaro, et al. (2019), we report an increase in grain Zn and Fe concentration due to AM colonization, and this led to lower molar ratios of PA to Zn and Fe in the AM Iride plants. This, in turn, implies that grain from the AM Iride plants generally had more bioavailable Zn and Fe than the grain of the mock-inoculated plants.

The PCA biplot illustrates that the grain samples across diverse durum wheat genotypes could, in general, be separated by AM fungal inoculation; the *R. irregularis*-inoculated plants generally had higher nutrient concentrations and more bioavailable Zn and Fe than the mock-inoculated plants. Positive effects of AM colonization on the bioavailability of Zn and Fe have also been reported for maize grain (colonized by *Glomus intraradices* TNAU-11–08) (Subramanian, Balakrishnan, & Senthil, 2013) and winter wheat grain (colonized by *Rhizophagus intraradices* BGC HEB07D or *Funnelliformis mosseae* BGC HEB02) (Ma, Luo, Li, & Wu, 2019). Furthermore, Ryan, McInerney, Record, and Angus (2008) showed in bread wheat grown in the field that AM colonization had a strong negative relationship with PA:Zn ratio, implying that greater root colonization may be linked to more bioavailable Zn. These contrasting results suggest that the impact of AM inoculation on PA accumulation in grain is complex, and dependent on many factors including crop species and genotype, AM fungal species and isolate, as well as the availability of nutrients in the soil including P, Zn, and Fe.

5 | CONCLUSIONS

To feed and nourish an increasing world population, it will be important to focus on breeding targets that, among other traits, improve micronutrient bioavailability in grain. We have demonstrated here that there is substantial genetic variation in grain micronutrients and phytate accumulation, which suggests there is potential to breed for increased micronutrients alongside decreased phytate. However, such adjustments to grain traits may negatively impact grain yield, and this should also be taken into account. There is also a need to consider how best to manage other soil-based factors, such as P (and other nutrient) application, and AM fungi.

Clearly, AM fungi affect plant P, Zn, and Fe uptake, and thus are highly interactive with the plant processes that ultimately contribute to grain micronutrient bioavailability. Further work to understand the mechanisms affected by AM colonization, whether they are direct or indirect, will enable us to begin teasing out this complex scenario. Future work will address this complex issue in a field setting with the indigenous soil microbial community intact.

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AUTHOR CONTRIBUTIONS

BTTT was involved in the design of the experiment, performed the research and data analysis, and contributed to data interpretation and writing the manuscript. TRC was involved in the design of the experiment, contributed to data interpretation, and writing the manuscript. JAA was involved in the design of the experiment, contributed to data analysis and interpretation, and wrote manuscript as it appears in its final form.
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**SUPPORTING INFORMATION**

Additional supporting information may be found in the Supporting Information section.

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