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An optimized high-throughput colorimetric assay for phytic acid quantification

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Abstract

Phytic acid (PA) is the primary storage form of phosphorus in seeds and is considered an anti-nutritional factor because of its ability to chelate essential minerals, thereby reducing their bioavailability. However, identifying low-PA mutants in large populations requires a cost-effective, accurate, and high-throughput screening method. A previously reported colorimetric method for quantifying PA in soybean (*Glycine max* (L.) Merr.). However, the throughput of that method is relatively low. In this study, we modified several key steps of the protocol to improve its throughput. The accuracy of the modified protocol was validated by comparing it with the original method and a commercially available PA quantification kit. The new high-throughput protocol showed high reproducibility and successfully distinguished existing low-PA mutants from their wild-type parent. The protocol was then used to screen a diversity panel of 202 pea accessions (*Pisum sativum* L.), which revealed a wide genetic variation in PA content. We identified two novel low-PA accessions, JI0383 and JI3253, with 69% and 48% reductions in PA, respectively, compared to the population mean values. This cost-effective method is expected to help researchers and breeders accelerate the development of low-PA crops to meet the current demand for high-quality plant-based foods.

Plain Language Summary

Seeds of many food crops, including pulses and cereals, contain relatively high concentrations of phytic acid (PA), also known as phytate. The presence of this compound in foods lowers their nutritional value because of its negative impact on the bioavailability of critical minerals such as iron and zinc. To develop low-PA cultivars, researchers and breeders require fast and inexpensive analytical methods to screen large numbers of samples from mutant populations, segregating populations, or natural diversity collections. This study aimed to improve the throughput of an

Abbreviations: HSD, honestly significant difference; ICP-OES, inductively coupled plasma-optical emission spectroscopy; PA, phytic acid; PCGIN, Pulse Crop Genetic Improvement Network; Pi, inorganic phosphate; SPDT, SULTR-like phosphorus distribution transporter.

existing PA quantification protocol proposed by modifying or eliminating steps that constrain its use in large-scale screening for PA content. The new optimized assay protocol was accurate, highly reproducible, and allowed the identification of two pea accessions with very low PA content.

1 | INTRODUCTION

Phytic acid (PA), or myo-inositol hexakisphosphate (InsP6), is the primary storage form of phosphorus in seeds, accounting for up to 80% of the total seed phosphorus (Bohn et al., 2008). PA plays an important role in plant physiology, particularly in seed germination, seedling development, and stress responses (Pramitha et al., 2021; Sparvoli & Cominelli, 2015). However, PA is considered an anti-nutritional factor in human and animal diets because of its ability to chelate essential minerals, such as iron and zinc, thereby reducing their bioavailability (Bangar et al., 2017; Moore et al., 2018; Sparvoli & Cominelli, 2015). Considering that many staple crops contain relatively high concentrations of PA (Pramitha et al., 2021; Wang et al., 2022), this exacerbates the global prevalence of micronutrient malnutrition in populations that depend on plant-based diets. Additionally, in high-income countries, shifting toward environmentally sustainable food systems requires increasing the consumption of pulses (Willett et al., 2019), which are naturally PA-rich and may lead to mineral deficiencies in specific demographic groups, notably women of reproductive age (Beal et al., 2023). Therefore, lowering PA concentration is necessary to unlock the nutritional and environmental benefits of pulse crops.

From a nutritional perspective, foods should ideally contain low levels of PA and high mineral density, particularly iron and zinc. However, achievements in developing staple food legumes with high mineral content have yielded only modest success (Bouis & Saltzman, 2017). One major challenge is that excessive mineral accumulation can trigger toxic responses in plants (Harrington et al., 2024), limiting the extent to which mineral concentrations can be increased without compromising agronomic performance. Thus, a practical breeding strategy would be to introduce low-PA alleles into agronomically superior cultivars that already possess a relatively high mineral content.

Screening large numbers of research materials, such as mutant populations or natural diversity collections, to identify low-PA variants requires an accurate and efficient method for quantifying PA. Several methods have been described for PA quantification, including anion exchange chromatography, high-performance liquid chromatography, and nuclear magnetic resonance spectroscopy (Gao et al., 2007). A major advantage of these methods is their ability to distinguish between InsP6 (PA) and lower inositol phosphates (≤InsP5) (Marolt & Kolar, 2020). However, these methods are expensive, require specialized equipment, and are unsuitable for high-throughput analyses. In contrast, cost-effective colorimetric methods that are amenable to higher throughput analysis have been developed. One such method is the molybdate blue assay, which is based on the reaction between phosphate and molybdate to form a blue complex. Using this principle, the commercially available Megazyme kit uses a combination of phytase enzymes to quantify total phosphate and PA-bound phosphate and is widely used for PA quantification (Cominelli et al., 2018; Gyani et al., 2020; Kumar et al., 2021). However, this method is relatively expensive and involves time-consuming sample handling steps. The molybdate blue assay has also been used to quantify the concentration of inorganic phosphate (Pi) and has facilitated the identification of several low-PA mutants in different crops (Gyani et al., 2020; Qamar et al., 2024; Warkentin et al., 2012). Because this method assumes that samples with high Pi are potential mutants with impaired PA biosynthesis, it may not detect mutants with reduced overall seed phosphate content caused by mutations affecting phosphate transport from lower plant tissues to developing seeds. For instance, in rice (*Oryza sativa* L.), Yamaji et al. (2017) showed significant reductions in grain Pi and PA concentrations in mutants of the *SULTR-like phosphorus distribution transporter* (*SPDT*), a key transporter that regulates phosphorus allocation to the grain. Therefore, the Pi-based molybdate blue assay alone is insufficient for identifying such valuable mutants. A third colorimetric method that directly measures PA is the Wade Reagent assay (Vaintraub & Lapteva, 1988), which was further modified by Gao et al. (2007). This method exploits the high affinity of PA for $Fe3^{+}$, where the pink color of the Fe^{3+}–sulfosalicylate complex (Wade Reagent) is depleted in proportion to the concentration of PA in the test sample. This offers a simpler and more cost-effective alternative that can be used for both quantitative and qualitative analyses of PA content. The method has been used for different crop species, including rice (Panda et al., 2023), wheat (*Triticum aestivum* L.) (Williams et al., 2024), chickpea (*Cicer arietinum* L.) (Basu et al., 2019), mung bean (*Vigna radiata* (L.) R. Wilczek) (Dhole & Reddy, 2016), and groundnut (*Arachis hypogaea* L.) (Hande et al., 2013). However, the utility of this method for large-scale screening studies is limited by its low throughput (Wen et al., 2022). Therefore, to improve throughput, this study aimed to further modify

and refine the colorimetric protocol proposed by Gao et al. (2007). All the steps of the modified high-throughput PA assay were performed in 96-well plates. This improved high-throughput protocol was highly reproducible and allowed the identification of novel low-PA pea accessions (*Pisum sativa* L.).

Core Ideas

- Previously developed methods for the quantification of phytic acid lack the throughput required to analyze a large number of samples.
- A new optimized version of the method proposed by Gao et al. (2007) was developed.
- The colorimetric assay is inexpensive, highly reproducible, and suitable for screening large numbers of samples.
- The new protocol facilitated the identification of two novel pea accessions, JI0383 and JI3253, with 69% and 48% lower PA, respectively.

2 | MATERIALS AND METHODS

Pea accessions were obtained from the John Innes Centre Germplasm Resource Unit (https://www.seedstor.ac.uk/). The Pulse Crop Genetic Improvement Network (PCGIN) diversity panel (https://pcgin.org/) consists of 230 lines covering the diversity spectra of the pea germplasm held at the John Innes Centre (Jing et al., 2010). In addition, two low-PA pea lines (*lpa* mutants), 1-150-81 and 1-2347-144, and their parental line (CDC Bronco) (Warkentin et al., 2012) were kindly provided by Professor Tom Warkentin of the University of Saskatchewan, Canada. The lines were grown in field plots at the Dorothea de Winton Field Station in Norwich in 2021. The final number of accessions for PA analysis was 202.

2.1 | Sample preparation

Approximately 10 g of dry pea seeds were ground using an MMT 40.1 Multi-use milling tube (IKA) for 1.5 min at 25,000 rpm. To ensure homogeneity, the samples were then sieved through a 0.250-mm mesh fitted to a custom-made frame. Subsequently, the samples were dried overnight at 70˚C.

2.2 | Chemical reagents

All reagents utilized in this study were of analytical grade and were sourced as follows: The PA/Total Phosphorus Kit (CAT#: 700004327) was obtained from Megazyme Inc.; PA sodium salt hydrate (CA#: P8810), ascorbic acid (CAT#: A7506), sulfuric acid (CAT#: 258105), ammonium molybdate tetrahydrate (CAT#: 09878), NaCl (CAT#: S9888), and trichloroacetic acid (CAT#: T8657) were sourced from Sigma-Aldrich; sulfosalicylic acid (CAT#: 11475533), nitric acid (CAT# 10149982), hydrogen peroxide (Fisher Scientific, CAT# 10530341), and HCl (CAT#: 10316380) were acquired from Fisher Scientific; and iron(III) chloride hexahydrate (CAT#: 31232) was obtained from Fluka.

2.3 | PA quantification

2.3.1 | Megazyme kit method

The principles underlying the method are described in McKie and McCleary (2019), and the reagents are available as a commercial kit (K-PHYT) from Megazyme. The assay was conducted according to the manufacturer's protocol (Megazyme Inc.). Briefly, 1 g of pea seed flour was weighed into 50-mL Falcon tubes and digested with 20 mL of 0.66 M HCl overnight. Subsequently, a 1 mL aliquot was transferred to a 1.5-mL tube and centrifuged at 15,000 × g for 10 min. Subsequently, 0.5 mL of the supernatant was neutralized with 0.5 mL of 0.75 M sodium hydroxide solution. In parallel steps, free phosphorus and total phosphorus were quantified, the latter of which was released through a two-step enzymatic dephosphorylation process. The molybdenum blue method was employed to determine phosphorus concentration, utilizing a standard calibration curve ranging from 0.125 to 8 μg/mL. The color intensity of the samples was measured at 655 nm using a microplate reader (ClarioStar, BMG LabTech), and the phytate-phosphate (PA-P) concentration was calculated according to the manufacturer's instructions.

2.3.2 | Gao et al. method

PA quantification in pea seeds was performed according to the method described by Gao et al. (2007) (see detailed diagram in Figure 1). Briefly, approximately 500 mg of seed flour was mixed with 14 mL of 0.64 M HCl overnight at room temperature. The tubes were centrifuged, and the supernatants were transferred into new tubes containing 1 g of NaCl to remove

FIGURE 1 A step-by-step comparison of the original protocol by Gao et al. (2007) and the modified high-throughput assay. In the modified method, omitted steps are indicated by a red "X," while other modifications are shown in bold text. The asterisk in step 2 indicates that Gao et al. (2007) did not specify how the samples were mixed; therefore, we mixed the samples by vortexing.

matrix components that could interfere with the colorimetric reaction (Gao et al., 2007). After incubation on ice for 1 h, the samples were centrifuged, and the clear supernatants were further diluted and used for analysis. The concentration of PA was determined using Wade Reagent, which was prepared by dissolving 300 mg of sulfosalicylic acid and 30 mg of ferric chloride hexahydrate in 100 mL of Milli-Q water. For color development, 3 mL of the sample was mixed with 1 mL of Wade Reagent, and the PA content was calculated from seven standard curve samples ranging from 20 to 80 µg/mL.

2.3.3 | Modified high-throughput PA method

The assay was conducted using 96-well plates rather than tubes (refer to the detailed protocol in Figure 1). Approximately 60 mg of seed flour was weighed into 1.2-mL eight-strip tubes arranged in a 96-well format (QIAGEN, CAT# 19560). Each plate comprised 88 pea samples, including duplicates of 1-150-81 and CDC Bronco, a known low-PA mutant and its parental line, respectively. The remaining wells were reserved for the standard curve and blank samples in the subsequent steps of the assay.

Using a 1-mL multichannel pipette, 800 µL of 0.64 M HCl was added to each sample. To mitigate clump formation, samples were gently mixed by pipetting while the acid solution was gradually added. Subsequently, the plates were sealed and positioned horizontally in beakers, which were then shaken overnight at 250 rpm at room temperature. The samples were centrifuged at 4000 rpm (~2720 × g) and 10˚C for 20 min. Thereafter, 250 µL of the supernatant was transferred to new 1.2-mL deep-well plates (STARLAB, CAT# E2896-0120),

and 250 µL of 5 M NaCl solution was added. The samples were incubated at 4˚C for 60 min, followed by centrifugation at 4000 rpm at 10˚C for 20 min. Finally, 40 µL of the clear supernatant was transferred to new plates and diluted to a 1:25 ratio using 460 µL of Milli-Q water. The colorimetric assay was performed in 96-well clear plates (Greiner, CAT# 655093) by combining 50 µL of Wade Reagent with 150 µL of the diluted samples, and the absorbance was measured at 500 nm.

2.4 | Elemental phosphorus analysis using inductively coupled plasma-optical emission spectroscopy

The concentration of total phosphorus was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES), as described in Harrington et al. (2023), with minor modifications. Approximately 100 mg of dried pea flour was digested with 1 mL of 68% nitric acid and 250 µL of 30%–32% hydrogen peroxide at 95˚C for 16 h. The final acid concentration was reduced to 0.85% by dilution with Milli-Q water. All samples were analyzed in duplicates and measured with three technical replicates. Each batch of samples contained a reference and pea sample, which were analyzed in duplicate across all runs.

2.5 | Data analysis

Analysis of variance was performed using R, and the significance of the differences between means was determined using Tukey's honestly significant difference test at $p \leq 0.05$.

3 | RESULTS

To develop a high-throughput assay for PA quantification, the amount of flour from seeds was downscaled from 500 to 60 mg, and the extraction volume was reduced from 10 to 0.8 mL (Figure 1). However, the most important modification of the original protocol was the replacement of solid NaCl with a 5 M solution form. This eliminates the need to weigh equal amounts of salt into tubes and the time required to dissolve it in step 6 of the original protocol (Figure 1). In addition, because 96-well plates are compatible with high-speed centrifuges, better separation of precipitates is achieved, which removes more sample matrix after salt treatment. Therefore, step 11 of sample mixing and centrifugation was omitted.

We validated the modified method by (i) comparing its performance with that of the original method and the commercial Megazyme kit and (ii) assessing its ability to distinguish the PA content profiles of *lpa* mutants from those of their wild-type parental line. All three methods clearly distinguished the *lpa* mutants (1-150-81 and 1-2347-144) from the wild-type Bronco CDC (Figure 2A). However, the PA content measured using the Megazyme kit was significantly lower than that measured using the Gao et al. (2007) method and the high-throughput protocol, both of which showed 27% higher PA values on average. Conversely, except for the wild type, the original protocol and the modified method were not statistically different, and their relatively higher PA values could be due to interference from unknown sample matrices, including Pi content. The Megazyme method measures only PA-P, whereas, according to Gao et al. (2007), the sample matrix, including its Pi content, may interfere with the Wade Reagent. Nonetheless, the magnitude of the difference between the mutants and the wild type remained nearly the same across the three methods, with a 48%–55% PA reduction in the mutants. In addition, the calibration curves were highly linear, with R^2 values of 1 and 0.99 for the Megazyme kit (Figure 2B) and Wade Reagent methods (Figure 2C), respectively. It is worth noting that the blank-corrected absorbance of the standards in the Wade Reagent is negative because the intensity of the assay color was inversely correlated with the concentration of PA.

To further test the utility of the modified protocol, we screened 202 accessions from the PCGIN pea diversity panel for their PA content. There was a wide genetic variation in PA content among the pea accessions, ranging from 5 to 21 mg/g, with an average of 16 mg/g (Figure 3A). The lowest PA was recorded in JI0383 and JI3253, with reductions of 69% and 48%, respectively, compared with the population mean. As expected, PA content was strongly positively correlated with total phosphorus (P) content (Figure 3B). The high agreement between P measured by ICP-OES and PA determined by the modified assay confirms that most of the P in pea seeds is stored in PA and that the high-throughput protocol can accurately quantify PA in pea seeds.

The reproducibility of the results obtained using the modified protocol was assessed by comparing the PA content in two lines, 1-150-81 and CDC Bronco, which were replicated in six 96-well plates and measured in three batches on different dates (Figure 4A). There was no significant difference between the plates, except plates C and F for PA content of 1-150-81. In addition, the coefficient of variation of the PA content across the plates was 7.9% and 6.7% for 1-150-81 and CDC Bronco, respectively. This indicates the high reproducibility of the data across plates and batches.

Finally, to confirm the low PA content of JI0383 and JI3253, we reanalyzed them along with two high-PA accessions using the Megazyme kit (Figure 4B). This confirmed that these lines were novel genetic variants with a very low PA content. In addition, the magnitude of the difference between the low- and high-PA lines was nearly the same when

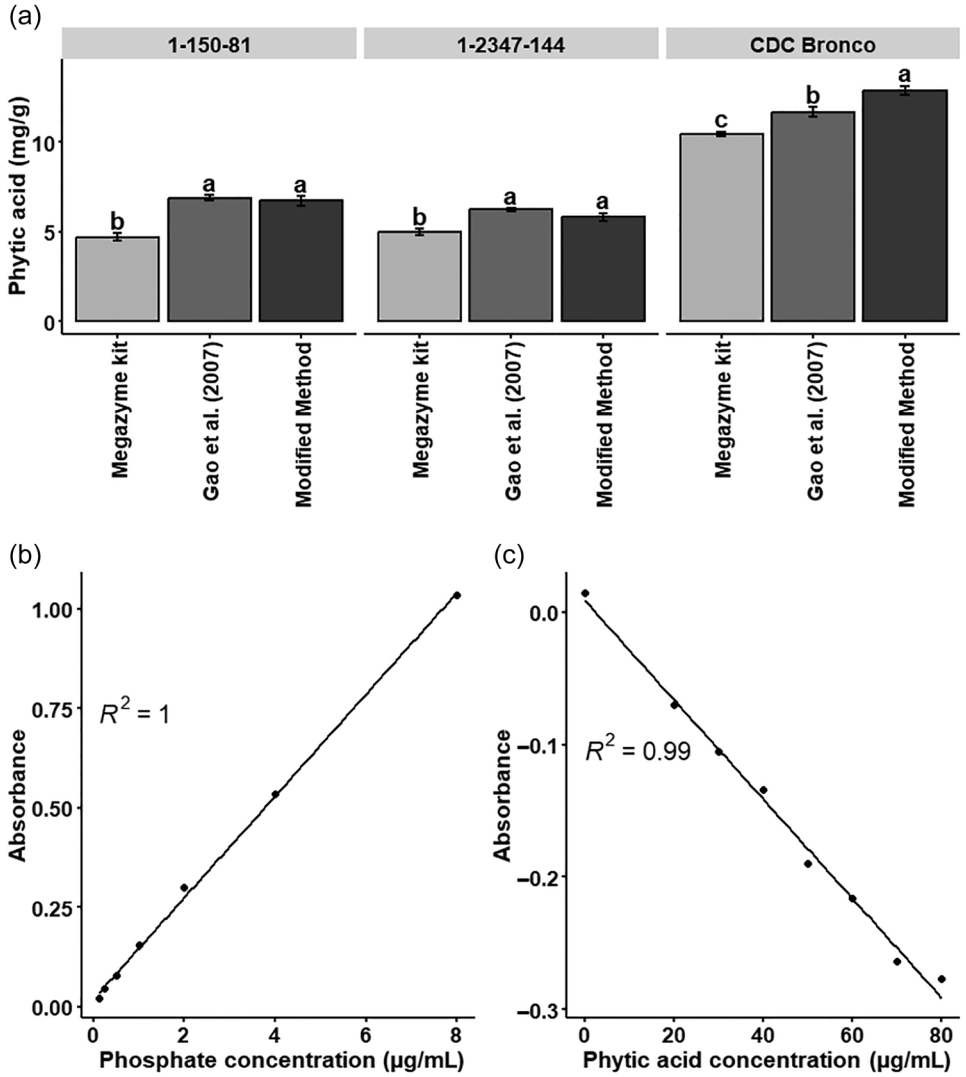

FIGURE 2 Phytic acid (PA) quantification using the Gao et al. (2007) protocol, the modified high-throughput method, and the commercial Megazyme kit. (A) Comparison of PA content of low-PA mutants and their parental line measured by the three protocols (a, b, and c). The data represent the mean of three replicates, and the bars represent standard errors. Tukey's honestly significant difference (HSD) mean comparison test was used, and bars with different letters indicate significant differences ($p \leq 0.05$). (B) and (C) show the ranges and R^2 values for the phosphate standard used in the Megazyme kit and the PA standard used in the Wade Reagent assay, respectively.

measured using the Megazyme kit and the modified assay. For instance, compared with JI0833 and JI0106, the reduction in PA in JI0383 was 80% using the Megazyme kit and 77% using the high-throughput assay method.

4 | DISCUSSION

Enhancing mineral bioavailability by lowering PA content in food crops can complement ongoing efforts to address the prevalence of malnutrition. Owing to its nutritional significance and the growing demand for plant-based foods, this trait is expected to be a major breeding objective. Several low-PA lines have been developed for many crops, including pea (Warkentin et al., 2012), soybean (*Glycine max* (L.) Merr.) (Gillman et al., 2009; Punjabi et al., 2018), common bean (*Phaseolus vulgaris* L.) (Cominelli et al., 2018), rice (Qamar et al., 2024; Tong et al., 2017; Yamaji et al., 2017), and maize (*Zea mays* L.) (Abhijith et al., 2020; Pilu et al., 2003). These materials are not widely available and are subject to intellectual property restrictions, necessitating the development of local genetic materials, either from natural diversity or through mutagenesis programs. Such endeavors require screening of large populations, and the available methods for PA quantification are expensive and have low throughput. In this study, we developed a refined version of the colorimetric method described by Gao et al. (2007). In the new protocol, we reduced the sample size and extraction volume by 10-fold and omitted or modified some steps to considerably improve the throughput. This modification is particularly valuable when

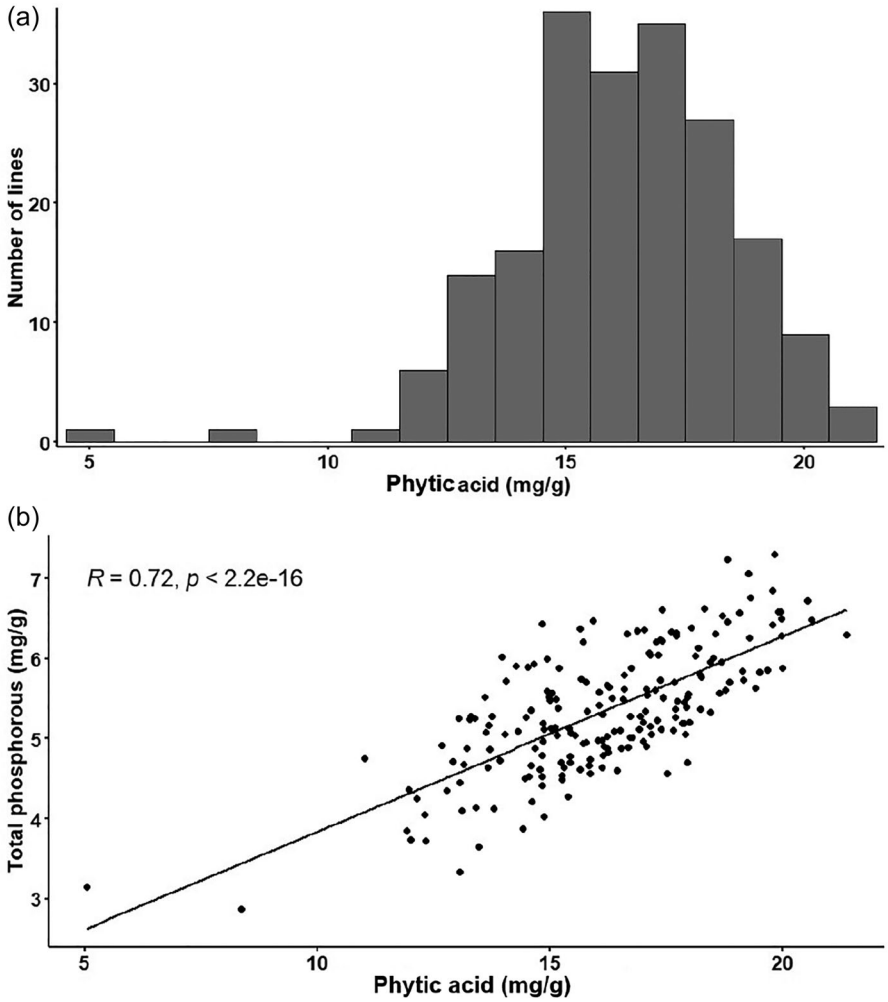

FIGURE 3 Variation in seed phytic acid (PA) content among 200 pea accessions screened using the high-throughput PA assay. Genetic diversity in pea for PA content (A) and its correlation with total phosphorus content measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (B).

working with limited seed quantities or when analyzing large populations.

By comparing the modified assay with the original protocol and the commercial Megazyme kit, we showed that these modifications did not compromise the accuracy of the protocol. The protocol can screen 528 samples every 2 days (six plates), assuming that the samples are already weighed in 96-well plates and one person handles the protocol. In the case of extremely large sample numbers, the throughput of the protocol can be further increased by grinding the seeds in 24, 48, or 96 batches using the Geno/Grinder 2010 Tissue Homogenizer (see different options at: https://cpsampleprep.com/). In addition, an electronic balance with the functionality to print data into an Excel file can enhance efficiency and minimize errors. In terms of the cost per sample, this method is extremely cheap and is a fraction of the cost of the Megazyme kit (50 assays).

Using the optimized protocol, we identified two pea accessions with very low PA content. Based on their history, the two accessions were not related. The JI0383 accession is a garden pea line with wrinkled seeds that was donated by Elsoms Seeds Ltd. to the John Innes Centre in 1968. JI3253, also known as Cameor, is a French field pea cultivar with round seeds that has been extensively used in pea genetic studies, including the first full genome sequence (Kreplak et al., 2019) and the creation of several genetic mapping populations (Ellis et al., 2023; Klein et al., 2020). Considering this breeding background and based on our own observations of the yield performance of these accessions both in the field and glasshouse, the loci conferring the low PA phenotype had no negative impact on the agronomic potential of these lines. This is crucial because of the agronomic penalties for PA reduction reported in pea (Warkentin et al., 2012), rice (Qamar et al., 2024; Zia ul et al., 2019), and maize (Abhijith et al., 2020). It appears that mutations in the PA biosynthesis pathway, which are often associated with the overaccumulation of Pi in the seed, have a negative impact on seed viability and germination. In contrast, Yamaji et al. (2017) showed

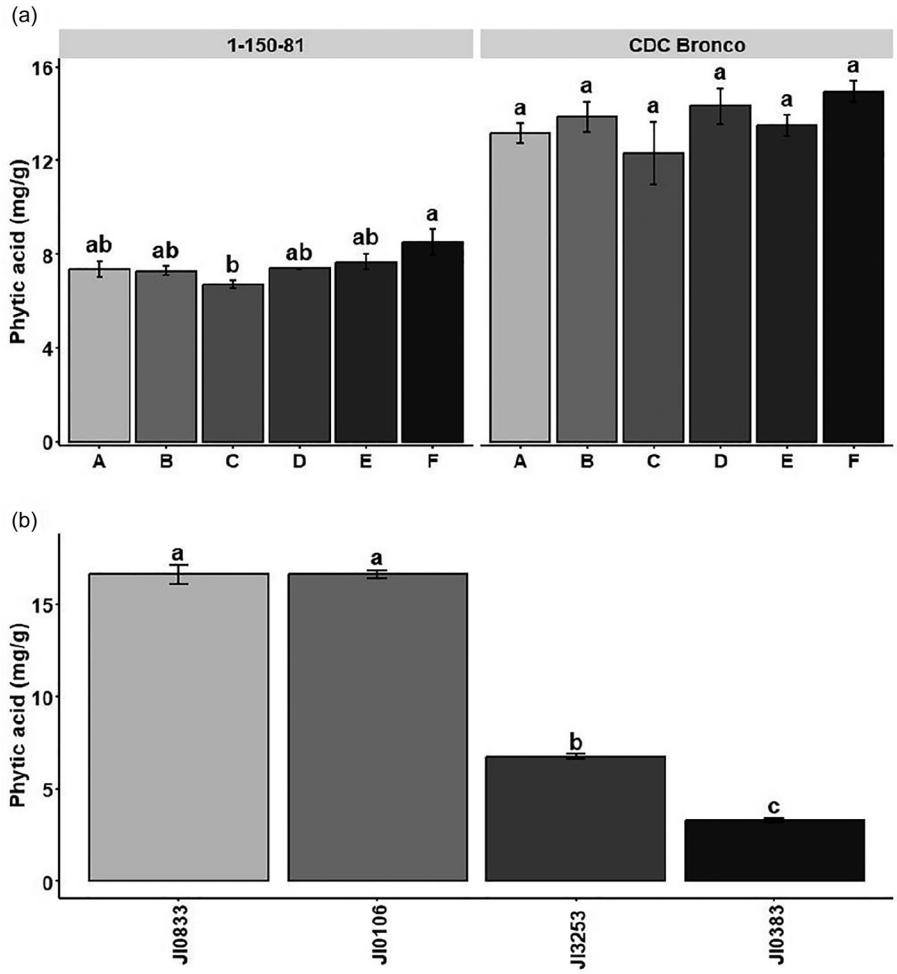

FIGURE 4 Reproducibility and accuracy of high-throughput assay. (A) phytic acid (PA) content of a low-PA pea mutant line (1-150-81) and its parental line measured in different plates (A–F) and batches (batch1 = A and B, batch2 = C and D, batch3 = E and F). (B) The PA content of the identified two low-PA lines and two high-PA lines that were reanalyzed using the Megazyme kit. Bars with different letters are significantly different ($p \leq 0.05$) according to Tukey's honestly significant difference (HSD) mean comparison test.

that mutating the rice SPDT, which is expressed in the xylem region of nodes and controls the allocation of phosphorus to the grain, significantly reduces PA without compromising yield and seed germination. Our preliminary data (not shown) showed that these two low-PA pea lines accumulated higher amounts of Pi in their leaves, which is characteristic of impaired phosphate transport from lower vegetative tissues to the grains (Yamaji et al., 2017). An in-depth genetic characterization of these low-PA lines is currently underway and will be reported in future publications.

In conclusion, the new protocol represents a significant advancement in seed PA quantification. Its high-throughput capability, cost-effectiveness, and reproducibility make it an invaluable tool for screening large populations in breeding programs aimed at developing low-PA crop varieties. The successful identification of novel low-PA accessions highlights the potential of this method for accelerating crop improvement efforts focused on enhancing the nutritional quality of legumes and other crops.

AUTHOR CONTRIBUTIONS

Ahmed O. Warsame: Conceptualization; data curation; formal analysis; methodology; visualization; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflicts of interest.

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