Assessing phytic acid variability, using high-performance thin-layer chromatography (HPTLC), on a diverse sample of *Lupinus luteus* L. (Fabaceae)

Evaluación de la variabilidad del ácido fítico, utilizando metodologías de cromatografía, en una muestra diversa de *Lupinus luteus* L. (Fabaceae)

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ABSTRACT

Phytic acid (PA) is a main phosphorus storage source for plants and accounts for ~75% of the seed total phosphorus (P). Due to its high mineral-chelating capacity, PA has been described as an antinutritional factor in human diets and animal feed, forcing artificial Pi (inorganic phosphorus) supplementation, and increasing phosphorus pollution through animal waste. However, studies have shown positive effects for human health, with potential anticancer activity. Thus, identifying variability in PA content in plant material has become a primary target for plant breeders. The aim of this study was to asses PA seed variation in a diverse sample of *L. luteus*. Eighteen yellow lupin accessions were analyzed using high performance thin layer chromatography (HPTLC) to provide insights on seed PA phenotypic variation. Chromatographic profiles only detected PA and Pi in mature seeds of *L. luteus* accessions, suggesting that other P-containing intermediaries are not accumulated in this legume species. Analyses of variance showed significant differences for PA and Pi seed content, indicating that accumulation of these compounds was influenced, at least in part, by *L. luteus* genotypes. PA and Pi seed content ranged from 13.97 to 24.37 mg $^{-1}$ and 1.27 to 1.68 mg g^{-1} (dry weight), respectively. PA significant phenotypic variation will not only aid the uncovering of possible plant metabolic pathways behind their accumulation, but also the identification of favorable variation to reduce antinutritional factors in yellow lupin.

RESUMEN

El ácido fítico (AF) es una fuente de almacenamiento de fósforo en plantas y representa ~75% del fósforo (P) total de la semilla. Debido a su capacidad quelante de minerales, AF ha sido descrito como un factor antinutricional en la alimentación de humanos y animales, forzando la suplementación artificial de Pi (fósforo inorgánico) en dietas, y aumentando la contaminación de fósforo a través de desechos animales. Sin embargo, estudios han demostrado efectos positivos sobre salud humana, dada su potencial actividad anticancerígena. Por ello, la identificación de variabilidad en el contenido de AF es de interés para los mejoradores de cultivos. El objetivo principal de esta investigación fue evaluar variabilidad de AF en una muestra diversa de *L. luteus*. Para ello, se estudió la variación fenotípica de AF en semillas de 18 accesiones, a través de cromatografía de capa fina de alto rendimiento (HPTLC). Perfiles cromatográficos sólo detectaron AF y Pi, sugiriendo que otros intermediarios no se acumularían en esta leguminosa. Análisis de varianza mostraron diferencias significativas para AF y Pi, lo que indicaría que la acumulación de estos compuestos es influida en parte por los genotipos de *L. luteus*. Los contenidos AF y Pi variaron entre 13,97 a 24,37 mg g^{-1} y 1,27 a 1,68 mg g^{-1} (base materia seca), respectivamente. La presencia de variación fenotípica significativa para AF no sólo proporciona información para el descubrimiento de las vías metabólicas envueltas en su síntesis, sino que además identifica variación favorable para la reducción de factores antinutricionales en lupino amarillo.

Palabras clave: ácido fítico, *Lupinus luteus*, antinutricional, HPTLC.

INTRODUCTION

Phytic acid (PA), or myo-inositol (1,2,3,4,5,6) -hexakisphosphate (IP6 or phytate), is a main phosphorus storage source in plant and animal cells (Bakewell, 2006). In plants, it has been associated to several cell functions, such as mobilizing an endomembrane store of Ca^{2+} in guard cells (Lemtiri-Chlieh *et al.*, 2000) and in signaling and response to plant pathogens (Murphy *et al.*, 2008). PA is found in several tissues and organs; however, it accumulates at large quantities only in seeds (Shi *et al.*, 2005). In general, PA accounts for

approximately 75% of the seed total phosphorus and represents more of 1% of the seed weight (Raboy *et al.*, 2001). Estimations of seed synthesized and stored phosphorous compounds have suggested PA acid corresponds to more of 60% of all phosphorous fertilizers sold annually world-wide (Lott *et al.*, 2000). Most PA is deposited in seed protein bodies, or globoids, as a mixed salt of mineral cations, such as K^+, Mg^{2+} and Zn^{2+} (Raboy *et al.*, 2001; Shi *et al.*, 2003). Due to its high mineral-chelating capacity, phytic acid has been described as an antinutritional in human and animal diets (Bentsink *et al.*, 2003; Gulewicz *et al.*, 2014; Petry *et al.*, 2014; Yorgancilar and Bilgiçli, 2014). When bound to phytic acid, minerals cannot be effectively absorbed by the intestine; and thus, they are largely excreted by humans and other non-ruminant animals, such as poultry, swine and fish (Raboy *et al.*, 2001). This negative attribute could affect human health by causing iron and zinc deficiencies, especially in developing countries where food is mainly seed-based (Mendoza *et al.*, 1998; Raboy *et al.*, 2001; Gibson, 2012). The low capacity of monogastric animals to digest PA reduces also the phosphorous intestinal intake forcing artificial Pi supplementation and increasing the contribution of phosphorus in animal waste and water pollution (Cromwell and Coffey, 1991).

Human health studies have shown PA has positive roles in human health (Rizvi *et al.*, 2006; Bozsik *et al.*, 2007; Lv *et al.*, 2015). For instance, both *in vivo* and *in vitro* studies have shown an anticancer activity in a variety of tumors (Silva and Bracarense, 2016), and cancers, such as melanoma (Rizvi *et al.*, 2006), leukemia (Bozsik *et al.*, 2007), breast (Dinicola *et al.*, 2016), and pancreatic cancer (Somasundar *et al.*, 2005). Phytic acid has also been described as a natural antioxidant (Bhowmik *et al.*, 2017), as a chemotherapeutic against hepatopathies (Abdel-Hamid *et al.*, 2007), reducing cardiometabolism risk (Bouchenak and Lamri-Senhadji, 2013), and as a neuroprotector against inflammatory responses associated to Parkinson disease (Lv *et al.*, 2015).

Natural variation for seed phytic acid content has been observed in *Arabidopsis thaliana* L. (Bentsink *et al.*, 2003), *Brassica rapa* L. (Zhao *et al.*, 2007), *Glycine max* L. (Israel *et al.*, 2006), *Phaseolus vulgaris* L. (Coelho *et al.*, 2002) and *Triticum aestivum* L. (Aggarwal *et al.*, 2015). Several QTLs have been identified in several crop species, most of them linked to anonymous markers (Larson *et al.*, 1998; Bentsink *et al.*, 2003; Zhao *et al.*, 2007). Low phytic acid (lpa) genotypes derived from mutagenesis experiments have allowed the identification of several key enzymes involved in the metabolic pathway of this trait (Shi *et al.*, 2003, 2005, 2007; Pilu *et al.*, 2005; Liu *et al.*, 2007; Sun *et al.*, 2007; Tagashira *et al.*, 2015). For instance, barley lpa1-1 mutant seeds show a decrease both in phytic acid and total phosphorous; however, several steps remain unclear (Raboy *et al.*, 2014). Nevertheless, the existence of natural diversity for PA seed content, and genomic regions explaining part of this variation, points out the presence of a strong genetic component behind this trait.

L. luteus is a member of the genistoid clade of the Fabaceae family (Mace *et al.*, 2008). The genus *Lupinus* comprises more than 200 annual and perennial herbaceous species of which several are cultivated and used as human food or animal feed (Gladstones, 1998). The major cultivated species are the old-world lupin *L. albus* L. (white lupin), *L. angustifolius* L. (narrow-leafed lupin) and *L. luteus* L. (yellow lupin), and the new world species *L. mutabilis* Sweet (pearl lupin or tarwii) (Wolko *et al.*, 2011).

Yellow lupine possesses several nutritional qualities and is characterized by having the highest protein content and twice as much cysteine and methionine than most legume species (Glencross *et al.*, 2004; Piornos *et al.*, 2015). However, antinutritional factors, such as alkaloids, alpha-galactosides, fiber, and phytic acid are found at high levels in this crop (Martínez-Villaluenga *et al.*, 2008; Adhikari *et al.*, 2012; Muzquiz *et al.*, 2012; Ertaş and Türker, 2014; Osorio *et al.*, 2018).

Thus, the main goal of this research was to asses PA phenotypic seed variability in a diverse sample of *L. luteus* using High Performance Thin Layer Chromatography (HPTLC) methodologies. HPTLC has been used to asses a wide range of SMs (Thenmozhi *et al.*, 2014; Agatonovic-Kustrin *et al.*, 2015; Kumar *et al.*, 2015; Osorio *et al.*, 2018), and it has shown to produce reliable and repeatable results in quantitative and qualitative yellow lupin secondary metabolite measurements (Osorio *et al.*, 2018).

MATERIAL AND METHODS

Plant material

Eighteen *L. luteus* accessions from different origins (Parra-González *et al.*, 2012; Osorio *et al.*, 2018) were included in the experiment (Additional Material: Table S1). Seeds from each accession were sown in September 2012 at CGNA's experimental fields (lat 38°41′S, long 72°25′W), Temuco, Chile. The experimental unit consisted of 1.2 m^2 plots seeded with 75 seeds. Three replicates of each accession were evaluated using a randomized complete block design. Seeds from 10 randomly selected plants were harvested, clean, dried for 72 h at 40 ˚C, and later manually dehulled. Freeze dried dehulled seeds were ground to fine powder using a MM200 mill (Retsch, Germany).

Chemicals and Reference compounds

Several chemical compounds were used as controls and standards to assess the content phytic acid in seeds. Phytic acid dodecasodium salt hydrate, D-myo-inositol 1,4,5-triphosphate, myo-inositol-2 mo-

nophosphate, potassium dihydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

PA extraction and HPTLC analysis

PA and inorganic seed P (Pi) were extracted as described by Aravena-Abarzúa, (2009) and Rasmussen and Hatzack (1998) with some modifications. Briefly, seed flours were extracted in 10% (w/v) TCA, 5 mM NaF and 5 mM EDTA for 15 min in an ultrasonic waterbath. PA and Pi were assessed using an HPTLC approach. Although HPTLC technologies have been used to quantify several secondary metabolites (Thenmozhi *et al.*, 2014; Agatonovic-Kustrin *et al.*, 2015; Kumar *et al.*, 2015; Osorio *et al.*, 2018), there is limited information with respect to PA assessment. However, previous studies (Aravena-Abarzúa, 2009) have validated their use when compared to standard colorimetric methodologies (Shi *et al.*, 2003).

Chromatography was carried out on cellulose glass HPTLC plates (20x10 cm, Merck, Germany). Samples were sprayed as bands of 8 mm using an ATS4 automated applicator (CAMAG, Muttenz, Switzerland). Distance between bands was 2 mm and bands started at 8 mm from the bottom of the plate. The sprayed volume was fixed at 0.8 µl for all samples. Plate development was carried out using an ADC2 automated development chamber (CAMAG, Muttenz, Switzerland) where chamber humidity was kept at 33% using an $MgCl_2$ saturated salt solution. Phytic acid chromatography involved a 10 min of tank saturation, with a solvent composition of 1-propanol, acetic acid, formic acid, water 8:2:9:1, and a migration distance of 60 mm. To allow detection, phytic acid and Pi (PO_4) were derivatized by spraying the plate with a methanol solution containing 8mM ammonium heptamolybdate, 0.1M HCl and 0.5M $HClO_4$. The plate was then exposed to UV light (366nm) for one minute, and blue bands (Hatzack and Rasmussen, 1999) scanned at 700nm. Quantification of phytic acid and orthophosphate were carried out correlating peak areas to the corresponding concentrations using a multilevel calibration and linear regressions (Osorio *et al.*, 2018). Quantifications, area peaks, and regressions were conducted using the Software WinCATS 1.4.4.6337 (CAMAG, Muttenz, Switzerland). Examples of plate, densitograms and linear regressions are provided in Figure 1.

Statistical analysis

Analyses of variances (ANOVA) were carried out to examine variation among genotypes for phytic acid content. Variances were analyzed using the GLM model from SAS 9.3 (SAS Institute, Cary NC). Least square means (lsmeans) were estimated for each accession, and phenotypic correlations between seed PA and Pi content was conducted using the CORR procedure (SAS 9.3, SAS Institute, Cary NC 2001).

RESULTS AND DISCUSSION

In most major cereal and legume crops, PA (myo-inositol(1, 2, 3, 4, 5, 6) hexakisphosphate or Ins P6) accumulates predominantly in seeds (Shi *et al.*, 2005; Murgia *et al.*, 2012), where represents ~75% of seed total P (Raboy *et al.*, 2001; Raboy, 2003). Our chromatographic (HPTLC) profiles only detected PA (Ins6) and Pi in mature seeds of *L. luteus* accessions (Figure 1), suggesting that other P-containing intermediaries, such as IP1, IP2, etc. are not accumulated in this legume species. Analyses of variance indicated significant differences for PA and Pi seed content across yellow lupin accessions (Table S1), suggesting that differential accumulation of these compounds was influenced by *L. luteus* genotypes. PA and Pi ranged from 13.97 to 24.37 mg g^{-1} and 1.27 to 1.68 mg g^{-1}, respectively (Figure 2). Evaluations carried out across lupin taxa have found differences among species with seed PA concentrations ranging from 3.12 to 19.8 mg g^{-1} (Dagnia *et al.*, 1992; De la Cuadra *et al.*, 1994; Aravena-Abarzúa, 2009), where *L. luteus* have shown the highest PA concentrations when compared with other lupin species (Petterson, 1998). Natural variation for seed PA content has been observed in several species, such as *Arabidopsis thaliana* (Bentsink *et al.*, 2003), *Brassica rapa* (Zhao *et al.*, 2007), *Glycine max* (Israel *et al.*, 2006) and *Phaseolus vulgaris* (Coelho *et al.*, 2002). Although differences for PA synthesis, or accumulation, may alter significantly seed P chemistry and composition, they usually have little effect on seed total P (Cichy and Raboy, 2009).

Although seed PA content has been explained by a genetic component, studies carried out across locations, and under different phosphorus fertilization levels, have shown a strong environmental effect (Saastamoinen, 1987; Dai *et al.*, 2007; Raboy *et al.*, 2014). Substantial variation has been observed for both inorganic P (Pi) and PA content in both leaf and seeds, where a main *Arabidopsis thaliana* QTL accounted for most of the Pi and PA differences in both tissues (Bentsink *et al.*, 2003). Comparisons among PA and Pi seed content did not show any clear pattern (Figure 3), as suggested by a low, and not significant, phenotypic correlation (r = 0.017; P-value = 0.93). This lack of correlation suggests that PA and Pi accumulation is uncoupled, and most likely under a different genetic control. Mutant recessive alleles in barley have reduced seed PA between 50% to 90% and greatly increase seed Pi, not resulting in changes in seed total P (Dorsch *et al.*, 2003; Ockenden *et al.*, 2004). Given that few studies have directly addressed the genetics of P transport into seeds (Raboy *et al.*, 2014), the limited amount of evaluations covering PA seed content in lupin species (Petterson, 1998), and the lack of discovered genes responsible of PA accumulation in *L. luteus*, more research should be conducted to uncover the basis of PA variation in this legume species.

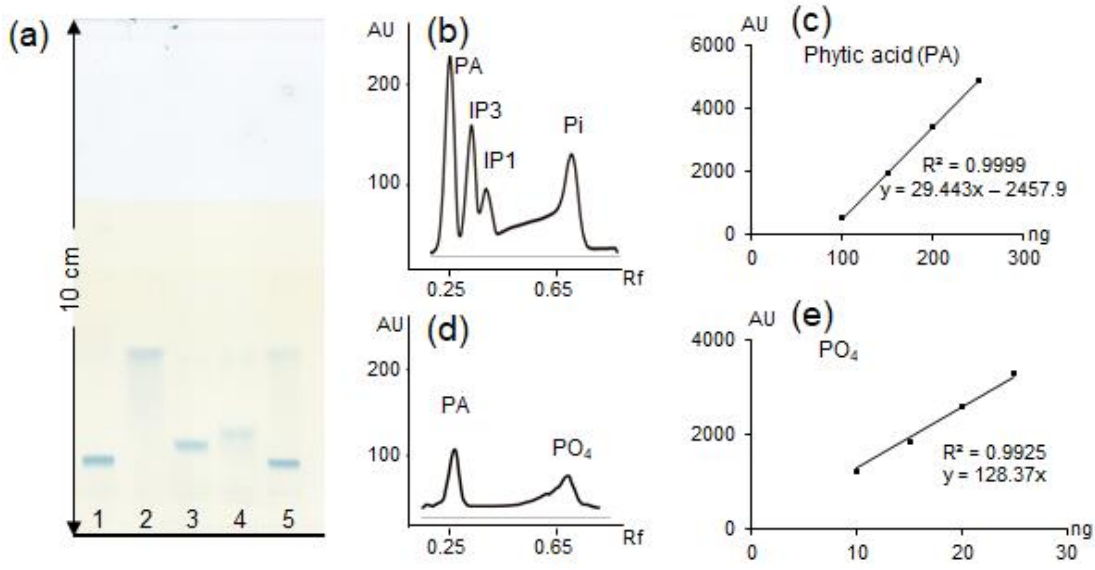

Figure 1. Chromatographic (HPTLC) PA, IP3, IP1 and Pi banding profiles, densitograms, and linear regression curves. (a) An example of a cellulose plate containing PA, IP3, IP1 and Pi controls and seed extract: 1, phytic acid (PA); 2, PO_4 (Pi); 3, inosytol triphosphate, (IP3); 4, inosytol monophosphate (IP1); 5, lupin seed extract. (b) and (d), HPTLC densitogram of phosphorus standards and lupin seed extract, respectively. (c) and (e), PA and Pi standard linear regressions, respectively.

Figura 1. Perfiles cromatográficos (HPTLC) para AF, IP3, IP1 y Pi, densitogramas, y curvas de regresión linear. (a) Ejemplo de un placa de celulosa conteniendo controles de AF, IP3, IP1, Pi y extractos de semilla: 1, Acido fítico (AF); 2, PO_4 (Pi); 3, inositol trifosfato (IP3); 4, inositol monofosfato (IP1); 5, extracto de semilla de lupino. (b) y (d), densitogramas de estándares y extracto de semillas, respectivamente. (c) y (e), regresiones lineares para los estándares FA y Pi, respectivamente.

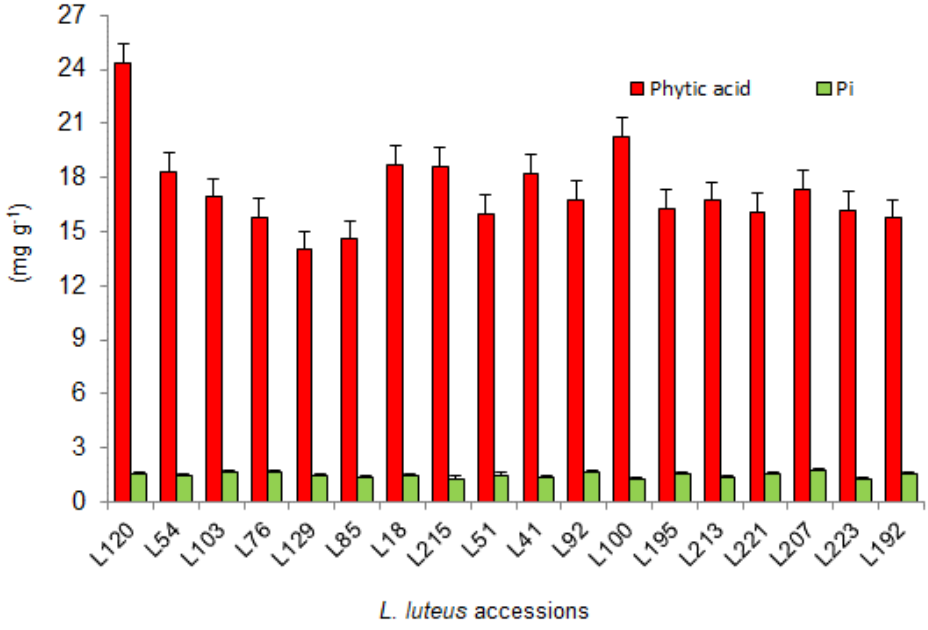

Figure 2. Phytic acid and Pi seed accumulation in *L. luteus*. Quantification, area peaks, and regressions were conducted using the Software WinCATS 1.4.4.6337 (CAMAG, Muttenz, Switzerland). Error bars correspond to standard errors.

Figure 2. Acumulación de ácido fítico y Pi en *L. luteus*. Las cuantificaciones, área de los picos, y regresiones fueron llevados a cabo utilizando el Programa WinCATS 1.4.4.6337 (CAMAG, Muttenz, Switzerland). Las barras de error corresponden a errores estándares.

CONCLUSIONS

Assessing PA diversity across *L. luteus* accessions provides a reservoir of phenotypic variation useful to aid the uncovering of possible plant metabolic pathways, and favorable variation to reduce antinutritional factors. In addition, by manipulating PA seed content, several yellow lupin derived products could aid human health, given its potential anticancer activity. Chromatographic evaluations found significant genotype effect for PA and Pi, suggesting that part of the observed phenotypic variation was under genetic control. In addition, the use of HPTLC methodologies could assess a wide-ranging pattern of diversity for PA, Pi, and other P containing metabolic compounds.

ADDITIONAL MATERIAL

Table S1. Level of significance (*P*) after carrying out analyses of variance for phytic acid and Pi in *L. luteus* seeds. Quantification of metabolites was conducted by high performance thin layer chromatography (HPTLC).

Cuadro S1. Niveles de significancia (P) para a análisis de varianza en el contenido de ácido fítico y Pi en semillas de *L. luteus*. La cuantificación de los metabolitos fue realizada utilizando cromatografía de capa fina de alto rendimiento (HPTLC).

Phosphorus compound	*P-value*
Phytic acid	<0.0001
Pi	<0.0001

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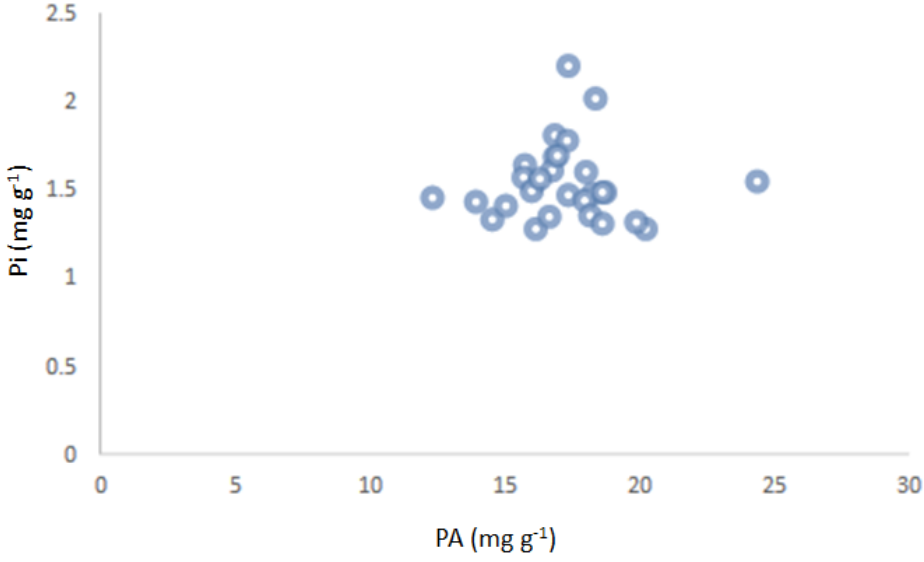

Figure 3. Comparisons between PA and Pi seed content in a diverse *L. luteus* sample. PA: phytic acid; Pi: PO_4.

Figura 3. Comparaciones entre el contenido de FA y Pi en semillas de una muestra diversa de *L. luteus*. FA: ácido fítico; Pi: PO_4.

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