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Nutrients' and Antinutrients' Seed Content in Common Bean (*Phaseolus vulgaris* L.) Lines Carrying Mutations Affecting Seed Composition

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Abstract: Lectins, phytic acid and condensed tannins exert major antinutritional effects in common bean when grains are consumed as a staple food. In addition, phaseolin, i.e., the major storage protein of the bean seed, is marginally digested when introduced in the raw form. Our breeding target was to adjust the nutrient/antinutrient balance of the bean seed for obtaining a plant food with improved nutritional value for human consumption. In this study, the seeds of twelve phytohaemagglutinin-E-free bean lines carrying the mutations *low phytic acid*, *phytohaemagglutinin-L-free*, *α-Amylase inhibitors-free*, *phaseolin-free,* and *reduced amount of condensed tannins*, introgressed and differently combined in seven genetic groups, were analyzed for their nutrient composition. Inedited characteristics, such as a strong positive correlation (+0.839 **) between the genetic combination "*Absence of phaseolin + Presence of the α-Amylase Inhibitors*" and the amount of "*accumulated iron* and *zinc*", were detected. Three lines carrying this genetic combination showed a much higher iron content than the baseline (+22.4%) and one of them in particular, achieved high level (+29.1%; 91.37 μg g^{-1}) without any specific breeding intervention. If confirmed by scientific verification, the association of these genetic traits might be usefully exploited for raising iron and zinc seed content in a bean biofortification breeding program.

Keywords: iron; zinc; biofortification; methionine; phaseolin; lectins; cellulose; phytic acid; saponins; condensed tannins

1. Introduction

Some substances contained in bean grains, such as lectins, phytic acid, condensed tannins, raffinosaccharides, sapogenols, are endowed with biological activities for health that can be beneficial or exert serious antinutritional effects. In well-fed populations, the health benefits afforded by most bean seed antinutritional compounds may be important against several human diseases [1–7]. On the other hand, when bean seed are utilized as a staple food (i.e., by African and Latin American people and by vegetarians), the antinutritional effect caused by several compounds can prevail, leading to a decrease in feed intake and growth rate, mainly due to micronutrient deficiency, especially iron and zinc [8–16]. The lectin proteins of the group of phytohaemagglutinins (PHAs; PHA-E and PHA-L) can reduce the bioavailability of dietary important micronutrients such as Fe and Zn [17,18] with negative effects on human health [19]. Phytohaemagglutinin binding to the gut wall is associated with a disruption of the brush border, reduced epithelial cell viability, hyperplasia in the crypts and increase in the weight of the tissue [20]. Phytohaemagglutinin damages the gut wall and causes coliform overgrowth in the lumen [21] and reduces the fractional rate of protein synthesis in skeletal muscle [22,23]. Among the four main lectins (PHA-E, PHA-L, Arcelin and the α-Amylase Inhibitors), PHA-E, and, in part, PHA-L, are the most dangerous for the health in mammalians. The elimination of the PHAs (PHA-E and PHA-L) from our bean materials is one of the important aims of our genetic improvement activity in bean

Phaseolin, the most represented seed storage protein of common bean, is another basic factor, studied for improving the nutritional quality of the seeds in this species. It was the object of breeding investigations, such as the development of *phaseolin-free* lines, aimed to raise the amount of methionine [24,25]. More recently, Montoya et al. (2010) [26] demonstrated that this protein is marginally digested in vitro and in vivo, above all due to its resistance to hydrolysis and/or proteolysis when assayed or introduced in the raw form. Unfortunately, the availability of common bean accessions or lines producing phaseolin-free seeds is not spread worldwide and, at present, they are used mainly for nutritional studies as it is done, for instance, at CREA-GB.

Another important group of compounds abundantly present in bean seeds and considered to have antinutritional properties, is represented by tannins and polyphenols [8,9,12,13]. These compounds are mostly present in the seed coat, especially in pigmented beans [13]. They can contribute to a 7–10% reduction in protein digestibility [11,27] and, above all, in reducing iron and zinc absorption at intestinal level [28,29]. In particular, Petry et al. (2010) [29] made particular experiments aimed to quantify the inhibitory effect of tannins on Fe bioavailability at the intestinal level. They demonstrated that the amount of absorbed iron was doubled when young women were feds with specific meals from which polyphenols were removed. It is important to state that, in this case, the absence/presence of polyphenols was tested on a basic meal that was dephytinized. Similar results were also observed in studies conducted in vitro (using Caco-2 cells) and in vivo (in poultries) [16]. In this experiment, the food based on white beans (low amount of polyphenols), provided significantly higher amounts of bioavailable iron than red beans, which contain more polyphenols.

Finally, the phytic acid is another important compound present in plant seeds. It has received considerable attention as a fibre-associated component with antinutritional effect. Literature describing the properties of this compound, especially those with antinutritional effects, is worldwide and so important that a Global Food Composition Database for Phytate has been published recently [30]. Many efforts in numerous directions have been made to contrast its antinutritional power [7,31]. However, according to many authors, the development of either *lpa* (low phytic acid) or biofortified varieties is the most sustainable approach in different species (genetic approach) [32,33]. Investigations carried out in vivo and aimed to quantify the real positive effect of the genetic phytate reduction on micronutrient absorption at intestinal level (i.e., by using meals based on seeds carrying *lpa* mutation), are rather limited. Using radioisotopes, Mendoza et al. (1998) [34], reported that iron absorption from meals based on *lpa* maize was significantly higher than from meals based on wild-type maize.

Hambidge et al. (2004 and 2005) [35,36] obtained the same results for zinc and calcium absorption, respectively, from meals based on *lpa* maize.

Based on the information reported in the literature until 1998, when we started our bean breeding activity, a high content of these substances in the bean seeds was considered negative for nutritional quality, suggesting to the breeders to plan new programs addressed to genetically reduce their amount. In this direction, we planned a breeding program aimed to modify the nutrients'/antinutrients' balance of seed content, and to study the physiological and/or biochemical effects on the interrelations between them. Our concept about this type of balance is based on the consideration that antinutrients are also important against several human diseases (as reported above) and, therefore, we consider that it is necessary to maintain a small amount of them (except PHAs that should be eliminated because it is very dangerous), although we do not know how much. Our breeding work started with the elimination of lectins from the bean seeds and continued with the introgression of the "reduced condensed tannins" trait, already available in nature in the white seed coat bean varieties. Firstly, we developed a few *lf* bean lines devoid of major lectin proteins (*lf* = *lectin-free* = absence of phytohaemagglutinin E-type (PHA-E), absence of α-Amylase Inhibitors (α-AI), absence of arcelin, but with PHA L-type (PHA-L) still present] [36]. Then we combined both *lf* and *wsc* (*white seed coat*, 98% reduction in tannins and polyphenols in the seed) traits to obtain the "*lf* + *wsc*" bean lines [37]. In a third step we started a new study focused to reduce the phytic acid. We isolated and characterized the *lpa* (low phytic acid) mutant (*lpa* 280–10) with a reduced amount of phytic acid (90% reduction) [38] which was introgressed in the "*lf* + *wsc*" beans to produce the new "*lf* + *lpa* + *wsc*" bean lines [39] with additive and combined effects. As soon as the new bean lines were available, the effect of these genetic modifications was studied by analyzing the seeds for the content of nutrients and antinutrients [40]. The accomplishment of this first study allowed us gathering new important and unexpected physiological and agronomic information, necessary for making new choices to be applied to future bean breeding programs. The genetic removal/reduction of the three antinutrients (main lectins, phytic acid and condensed tannins) led to a reduction of other antinutrients such as lignin and saponins, and to a strong increase of nutrients such as crude proteins and total zinc (30% each), and free Pi (600%) [40]. In addition, the in vitro iron bioavailability, as measured via a Caco-2 cell model [41], resulted on average twelve times higher in the "*lf* + *lpa* + *wsc*" bean seeds than in the wild type (*wt*) coloured parents [40]. One of the *lpa* beans described in this last work, namely the line 586/8X87-brown, was later tested in nutrition experiments conducted on young women by Petry et al. (2013) [42]. In particular, these authors demonstrated that reducing phytic acid by more than 90%, iron absorption was significantly increased from 60% to 163%, whereas the polyphenol concentration in the presence of phytic acid, unexpectedly, did not influence the iron bioavailability. In the next work, Petry et al. (2014) [43] again demonstrated the negative influence of phytic acid on iron absorption from biofortified beans in Rwandese women with low iron status. In particular, the showed that the extra amount of iron bred into the beans is of relatively low bioavailability, and that currently available biofortified bean varieties provide only a small extra amount of absorbable iron compared to normal beans.

In the present research, we compared the chemical composition in terms of nutrient/antinutrient seed content of twelve newly developed bean lines (plus a control) carrying five distinct mutations on genes affecting the accumulation of specific nutrient/antinutrient compounds, introgressed and differently combined in seven genetic groups of lines (genetic combinations). The seed composition of all materials was analyzed in order to: (1) find out the possible presence of new differences and/or new interrelations in the nutrient/antinutrient content, generated by all main genetic traits introgressed, in particular, in five genetic combinations never studied; (2) examine the interrelation existing between four theoretical genetic variables vs. all other quantitative variables chemically analyzed. The basic aim of the present study was to gather information in common bean on the interrelations existing between the different nutritional and antinutritional variables in order to understand how they can be usefully modified at genetic/physiological level and exploited in the direction of human needs.

2. Materials and Methods

2.1. Term Definitions

The terms "genetic group" or "genetic combination" are used in this work to define the whole of specific genetic characteristics, carried singly or in combination by the plants of a bean line or cultivar (cv), whose effects on nutrient/antinutrient content are the object of the present study. This definition is made to distinguish these terms from that of "genetic background" (also used in this text and commonly applied by the scientific community) that indicates the total genetic characteristics carried by an individual or group of individuals.

2.2. Plant Material

The seeds produced by twelve bean lines in which five mutations were introgressed and differently combined in seven genetic groups were the object of this study. A list of the evaluated lines, including the cv BAT 881 (eighth genetic group) used as the control with related genetic traits, is summarized in Table 1.

The origin, main steps of development and other information on all materials, are reported in Table A1 (see Appendix A). Photoperiod requirement for all lines is neutral. Except the cv BAT 881, all the twelve bred lines were devoid of the main lectin protein PHA-E, which is very dangerous for mammalians' health if introduced in the raw form [21–23]. The generation level reached through progressive selection activity after the last cross, was F7 for the line HP5/1, and over F8 for all other lines (cv BAT 881 included).

The following mutations were introgressed: (1) The absence of all major lectin proteins except PHA-L (introgressed from the CIAT accession G6388–CIAT, Centro Internacional de Agricultura Tropical, Cali, Colombia; see also CGIAR–www.cgiar.org); (2) The absence of all major lectins except "Pinto bean lectin + α-Amylase Inhibitors (α-AI)" (introgressed from the bean accession "Pinto UI 111"); (3) Absence of *phaseolin*, the major protein accumulated in the bean seed; (4) *lpa* (low phytic acid −90% reduction); (5) *wsc* (white seed coat −98% reduction of condensed tannins). Being products of the same genetic locus, the presence of PHA-L excluded the presence of (Pinto lectin + α-AI) and vice versa.

The seeds submitted to chemical analyses were all produced from plants grown in a field located at Casalecchio di Reno (Bologna province, Italy), cultivated according to the organic farming system.

2.3. Chemical Analyses of the Seeds

The seeds of the materials listed in Table 1 were submitted to the following quantitative compositional analyses: (1) dry matter (DM), (2) compounds exhibiting nutritional effect on human and animal growth [crude proteins, total starch, cystine and methionine, the polyamine spermidine, total iron and total zinc, total minerals (ash)], and (3) compounds exhibiting antinutritional effect on human and animal growth [trypsin inhibitors, condensed tannins, total phenolics, three sapogenols (oleanolic acid, soyasapogenol A and soyasapogenol B), fiber components (hemicellulose, cellulose and lignin)]. In addition, two new variables were examined, namely, the in vitro starch digestion (expressed as starch hydrolysis index, HI), and the in vitro protein digestibility (% of proteins digested at 180 min of incubation). Being potential indices of starch and protein utilization, these variables were reported in the Tables and submitted to statistical analyses in the same way as the values of nutrient/antinutrient factors. BAT881 is the control (*wild type–wt*) cultivar for the content of all nutrient/antinutrient compounds examined.

Table 1. Bean materials (the control cultivar BAT881 and twelve developed lines) and related genetic traits evaluated for seed nutritional conposition.

No.	Genotype	Presence of the *lpa* Mutation	*	Phaseolin	*	Seed Lectins [a]	*	Phaseolin Absence + α-AI Presence	*	Seed Coat Color	Plant Growth Habit [b]	Genus of Origin
1	HP5/1	*lpa*	1	absent	1	PHA-L (*lf*)	0	0		brown	det. 1b	*P.vulgaris* + *P. coccineus*
2	730/1	*wt*	0	"	1	"	0	0		dark mottled	"	"
3	042/5	"	0	"	1	Pinto bean lectin + α-AI	1	1		red mottled	det. 1a	"
4	041/1	"	0	"	1	"	1	1		purple mottled	"	"
5	720/12	"	0	"	1	"	1	1		" "	"	"
6	WH1/28	*lpa*	1	*wt*	0	PHA-L (*lf*)	0	0		white	det. 1a	*P.vulgaris*
7	WH3/76	"	1	"	0	"	0	0		" "	det. 1b	"
8	WH4/84	"	1	"	0	"	0	0		" "	"	*P.vulgaris* + *P. coccineus*
9	BR2/11	"	1	"	0	"	0	0		brown	"	*P.vulgaris*
10	938	*wt*	0	"	0	"	0	0		brown	det. 1a	*P.vulgaris*
11	720/18	"	0	"	0	Pinto bean lectin + α-AI	1	0		brown mottled	"	*P.vulgaris* + *P. coccineus*
12	720/20	"	0	"	0	"	1	0		red mottled	"	"
13	BAT 881	"	0	"	0	PHA-E + PHA-L + α-AI	1	0		brown	det. 1b	CIAT cv

Abbreviations: PHA = phytohaemagglutinin; α-AI = alpha-Amylase Inhibitors; *wt* = wild type; *lpa* = low phytic acid mutation (phytic acid reduced to 10%); *lf* = lectin-free; det. = determinate; CIAT = Centro Internacional de Agricultura Tropical, Cali, Colombia, see also CGIAR (www.cgiar.org). (a) Related to the lectin proteins present in major amount only (PHA-E, PHA-L, Pinto bean lectin, and α-AI). Arcelin was not present in anyone of the parents used in the breeding program; (b) plant growth habit according to Singh et al. (1982) [44]. PHA-L (*lf*) = PHA-L is the only lectin protein still present in these beans, in the past considered lectin-free. (*) Theoretical variables showing presence/absence of the genetic traits reported above: [*Presence of the* lpa *mutation*, *Absence of phaseolin*, *Presence of the α-AI* in the seed lectins, and a combination of two of them (*Absence of phaseolin + Presence of the α-AI*)]. For each one of the four theoretical variables, based on the plant characteristics reported in the previous adjoining column/s, the value "1" was attributed to the genotypes carrying the genetic trait (or combination of them) whose effect were put under observation, whereas, the value "0" was given to all other genotypes. Each of these theoretical variables was correlated vs. the quantitative ones of Tables 3, 4, and 5 (see "*Second group of correlation analyses*" reported in M&m). (") This symbol means '*As reported above*'.

2.3.1. Dry Matter (DM), Fiber Components (Hemicellulose, Cellulose and Lignin), Ash, Total Phenolics and Condensed Tannins

Analyses were conducted on samples dried at 60 °C to constant weight and ground in a Cyclotec Model 1093 sampling mill (Foss Tecator AB, Höganäs, Sweden) through a 1 mm sieve. Dry matter (DM) was determined drying the samples in a forced-air oven at 105 °C for 12 h. Ash were determined after incineration of the samples at 495 °C for 5 h. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to the method of Goering and Van Soest (1976) [45]. Cellulose was estimated by subtracting ADL from ADF and hemicellulose by subtracting ADF from NDF values [46,47]. No correction for ash was made. The content of total phenolics in plant extracts, obtained by treating 50 mg of ground seed samples with 2 mL of 70% MeOH solution, was determined using Folin-Ciocalteu reagent [48] according to the method reported by Taga et al. (1984) [49]. All results were expressed as mg gallic acid equivalents g^{-1} dry material. Total proanthocyanidins (condensed tannins) were determined by the butanol/HCl method, as detailed by Porter et al. (1986) [50]. All results were expressed as mg delphinidin equivalents g^{-1} dry material. Each datum, submitted to statistical analysis, was the mean of three absorbance values (replications).

2.3.2. Sapogenols

Sapogenins were quantified by an internal standard method as reported in Tava et al. (1993) [51]. Briefly, 250 mg of ground seed samples were treated with 30 mL of HCl 2N in 50% MeOH under reflux for 8 h. Betulinol (0.2 mg) was added as internal standard, MeOH was removed under vacuum and sapogenins were extracted with ethyl acetate (3 × 15 mL). The organic solution was dried over anhydrous Na_2SO_4 and evaporated until dry. Sapogenins were identified by GC/MS and quantified by GC/FID as their methyl-trimethylsilyl derivatives. Soyasapogenol B was identified and quantified based on all soyasapogenol artifacts obtained under acidic condition of hydrolyses as described by Tava et al. (2017) [52]. GC/FID analysis was carried out using a Perkin-Elmer model 8500 GC equipped with a 30 m × 0.32 mm i.d. × 0.25 μm, DB-5 capillary column. Injector and detector temperatures were set at 350 °C and the oven temperature programme was 90 °C for 5 min, increased at 20 °C/min to 250 °C for 1 min and then increased at 4 °C/min to 350 °C for 15 min. Samples (1 μL) were injected in 'splitless' mode. Helium was the carrier gas with a head pressure of 12.3 psi. GC/MS analyses were carried out using a Perkin-Elmer Clarus GC equipped with a MS detector and a 30 m × 0. 25 mm i.d. × 0.25 μm, Elite-5MS capillary column using the same chromatographic conditions as for GC/FID. Mass spectra were acquired over 50–850 amu range at 1 scan/sec with ionizing electron energy of 70 eV; transfer line 310 °C, carrier gas He at 1.2 mL/min. The standard calibration graph was obtained using pure soyasaponin I as representative of soyasapogenol B saponins and analyzed by GC/FID using betulinol as internal standard. Results are reported in Table 5 as the mean of three independent determinations ± SD.

2.3.3. Trypsin Inhibitor Activity

It was measured following the AACC Method 22–40.01 (1999) [53]. Different volumes (50, 100, 150 μL) of seed extracts (500 mg of flour in 100 vol of H_2O) were incubated with a fixed amount (5 μg) of trypsin (Sigma T-8642) for 5 min at 37 °C, then the reaction was stopped by adding 500 μL of BApNA (benzoyl-dl arginine-p-nitroanalide) reagent (Sigma B-3279) and 100 μL of acetic acid and OD410 nm was measured. Inhibitory activity was expressed as μg of trypsin inhibited per mg of flour.

2.3.4. Total Iron (Fe) and Total Zinc (Zn) Content Evaluation

Five grams of flour were dry-ashed and mineralized with five mL of HNO_3 65% and 1 mL of H_2O_2 120/130 volumes in a microwave oven as follows: (1) two five-minute steps at 400 watt, raising the temperature to 70 °C; (2) two five-minute steps at 560 watt, temperature to 125 °C; (3) two five-minute steps at 800 watt, raising the temperature to 170 °C. After cooling, each sample was filtered in a

graduated round-bottomed flask transferring the material by means of small-volume washings with purified milli-Q water until the final volume of 25 mL was reached. Samples were then read in an optical ICP (Inductive Coupled Plasma) Spectrometer Perkin Elmer Optima 2100 DV.

2.3.5. Crude Protein, Total Starch, Cystine and Methionine

All samples were assayed in duplicate according to the AOAC (2000) [54] for crude protein content (CP method 976.05). Total starch content was determined by applying an enzymatic method according to the procedure as detailed by Masoero et al. (2010) [55]. Amino acids analysis was conducted with an Automatic Amino Acid Analyser (model 3A29; Carlo Erba, Italy).

2.3.6. Protein Digestibility

Protein digestibility was measured in vitro following the method of Njintang et al. (2001) [56]. Briefly, 1.0 g of ground flour sample was suspended into 35 mL of 0.1 N HCl and incubated for 5 min in a shaking water bath at 37 °C. The pH of the mixture was then adjusted to 2. This blend was then transferred into dialysis tubing, 5 mL of freshly prepared pepsin (Sigma P-7000, Sigma–Aldrich®Co, Milan, Italy) enzyme solution (7 mg mL^{-1}) was added. The tubes were introduced into a beaker (placed in a shaking water bath at 37 °C) containing 400 mL of 0.1 N HCl. Aliquots from dialysate samples were collected at 180 min of in vitro incubation and analyzed for CP content [54]. For each sample, the in vitro protein digestibility was expressed as a percentage of the crude protein content of the corresponding raw sample before digestion.

2.3.7. In Vitro Starch Digestion

The in vitro starch digestion was performed as detailed by Giuberti et al. (2012) [57]. The protocol involves a two-steps hydrolysis simulating gastric and pancreatic human phases. Samples were prepared in glass tubes containing glass balls and pre-treated with a 0.05 M HCl solution (5 mL) plus pepsin (5 mg mL^{-1}; Sigma P-7000, Sigma–Aldrich®Co, Milan, Italy) for 30 min at 37 °C under agitation. The pH was adjusted to 5.2 by the addition of 0.1 M sodium acetate buffer, and then, 5 mL of an enzyme mixture with an amylase activity of about 7000 U/mL given by pancreatin (about 7500 FIP-U/g; Merck 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (about 300 U/mL; Sigma A-7095, Sigma–Aldrich®Co, Milan, Italy) and invertase (about 300 U/g; Sigma I-4504, Sigma–Aldrich®Co, Milan, Italy) were added. Milled white bread (1 mm screen; 723 g/kg dry matter starch content) was used as a reference sample for hydrolysis index (HI) calculation (details in below). Aliquots (0.5 mL) were taken from each tube at 0 (prior to the addition of the enzyme mixture simulating the pancreatic phase) and at 30, 60, 120 and 180 min after the enzyme addition, then absolute ethanol was added and the amount of the released glucose was determined colorimetrically with a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy). The proportion of the in vitro digested starch was calculated using a factor of 0.9. The HI was obtained from the ratio between areas under the in vitro digestion curve up to 180 min for each sample and the corresponding area for wheat bread (WB) was used as the reference (considering the HI of WB 100 by definition) [57].

2.3.8. Spermidine

Spermidine was extracted according to the following protocol: 0.25 g of dried and ground samples were extracted in 1.5 mL of 75% (*v*/*v*) methanol containing 0.05% (*v*/*v*) trifluoroacetic acid (TFA). After homogenization, the samples were stirred for 40 min and centrifuged at 19.000× *g* for 10 min. The extracts were filtered through 0.2 µm polytetrafluoroethylene (PTFE) filters. Each breeding line was extracted twice, resulting in a total of 26 extracts; each extract was analyzed twice.

All the extracts were analyzed using reversed phase liquid chromatography coupled to a photodiode array detector and to an ion trap mass spectrometry (LC–PDA–MS) system. Such a system consisted of an ultra-performance liquid chromatography (UPLC) DIONEX Ultimate 3000 model coupled to an LTQ XL mass spectrometer (Thermofisher Scientific, Sunnyvale, CA). A 5 µL aliquot

of sample was injected on a Luna C18 (100 × 2.0 mm, 2.5 µm particle size) column equipped with a Security Guard column (3.0 × 4.0 mm) from Phenomenex (Torrance, CA, USA). The separations were carried out using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% (*v*/*v*) formic acid, with a flow rate of 0.220 mL min^{-1}. The initial solvent composition consisted of 95% (*v*/*v*) of A and 5% (*v*/*v*) of B increased linearly to 25% A and 75% B in 25 min and was maintained for 1 min, and then returned to 95% of A in 1 min. The column was equilibrated to 95% A and 5% B for 11 min before the next injection. The analysis lasted for 38 min and the column temperature was set to 40 °C. Mass spectra were obtained in positive ion mode over the range *m*/*z* 70–1.400. The capillary voltages were set at 9.95 V and the source temperature was 34 °C. Quantitative determination of the compound was conducted by comparison with dose response curve based on *m*/*z* data from authentic and appropriately diluted standard solution of Spermidine purchased from Sigma-Aldrich (St. Louis, MO). Xcalibur software version 3.0 (Thermofisher Scientific, Sunnyvale, CA, USA) was used to control all instruments and for data acquisition and data analysis.

2.4. Statistical Analysis of the Data

The data of ash and trypsin inhibitors were recorded without replications and, therefore, they were not subjected to statistical analysis. Condensed tannins, total phenolics, hemicellulose, cellulose, lignin and spermidine were analyzed in triplicate, whereas the other variables were analyzed in duplicate. The genotypic values of each variable were the means calculated between the replicated data.

2.4.1. Assessment of Preliminary Conditions for the Applicability of the Analysis of Variance

Differences among lines for all recorded traits were assessed by analysis of variance (ANOVA). Prior to this analysis, the preliminary conditions for the applicability of ANOVA were verified. The normality of data distribution was checked and, when necessary and possible, adiusted (data tranformation) by applying the *skewness and kurtosis test* (Free Statistics Software Calculator-*https://www.wessa.net/rwasp_skewness_kurtosis.wasp#output*) to the data of each one of the 19 variables examined.

The *Bartlett's test* was then applied to both raw and transformed data to verify the assumption of homogeneity of variances (homoscedasticity) between the samples. The test was run on the SAS software according to Donatelli and Annicchiarico (1990) [58].

Finally, we performed the *Shapiro-Wilk* test to assess the normality of the residual distribution. The test was obtained as a part of the "UNIVARIATE" Procedure in the SAS software, version 8c (SAS Institute Inc., Cary, NC, USA).

2.4.2. Analysis of Variance (ANOVA)

All the variables that satisfied the homoscedasticity and normality conditions were submitted to ANOVA–One factor complete randomized block design model analysis [MSTAT–C software, version 1.42, (Michigan State University, East Lansing, MI 48824, USA), FACTOR function]. For each variable, the analysis assessed the effect and computed the respective probability (P value) exerted by the two factors replication (effect not shown in Tables) and cultivar/lines.

The Duncan's multiple range test (MSTAT–C software, version 1.42, RANGE function) was applied to compare line/cultivar mean values at a significance level set for a $p \leq 0.01$. It was applied only when the *F* test from the ANOVA was significant.

2.4.3. Non-Parametric Test

The data of the variables that did not satisfy the preliminary conditions were submitted to the non-parametric *Kruskal-Wallis test*, whose chi-square value (χ^2) gave an estimation of the among-line variation. The test was performed by the "NPAR1WAY" Procedure of the SAS software, version 8c.

2.4.4. Correlation Analyses

The data of the twenty-one quantitative variables reported in Tables 3, 4 and 5 were submitted to two groups of simple correlation analyses (MSTAT–C software, version 1.42, CORR function) to identify possible interrelations. The first group (210 correlations) was performed between each variable vs. all others. The second one (84 correlations), was performed between each one of the 21 variables of Tables 3, 4 and 5 vs. four new theoretical variables reported in Table 1 whose values could reproduce four genetic/physiological conditions in the seeds. Three of these genetic conditions were: (1) *presence of the* lpa *mutation*; (2) *absence of phaseolin*; (3) *the presence of the α-AI*. The fourth additional condition examined was given by the combined presence of two traits in the same genetic combination: *Absence of phaseolin + Presence of the α-AI*. All genetic conditions were according to the characteristics reported in Table 1 for every line/cv. The expressions of all the physiological conditions are under strict genetic control.

3. Results

3.1. Assessment of Preliminary Conditions for the Applicability of the Analysis of Variance

In Table 2 we reported the summary of the assesment of preliminary conditions for the applicability of the analysis of variance for all recorded variables on the set of thirteen common bean lines examined. In the same Table we reported also the results of a non-parametric assessment (*Kruskall-Wallis test*) showing the variation present among lines for the variables not complying with the preliminary conditions for the applicability of the ANOVA analysis.

3.2. Tables 3–5 Content

In Tables 3–5 we reported: (a) the unreplicated genotypic values for ash and trypsin inhibitors; (b) genotypic means and standard deviations (SD) calculated on the genotypic raw data, and results of the Duncan's test for each genotype of each variable subjected to ANOVA; (c) maximum and minimum values, including their ratio (max/min), all calculated on the thirteen genotypic data of each variable; (d) coefficient of variation (%) calculated on the replicated data of each of the 19 variables statistically analyzed. Its value, obtained as an output from the ANOVA, is = (SD/X) × 100, where SD is the standard deviation of the replicated data of a variable, and X is the arithmetical mean of the variable data.

3.3. Analyses of Seeds: Nutrient and Antinutrient Compounds

Data on chemical composition, and related results of statistical analysis of the variables exhibiting nutritional effect, are shown in Tables 3 and 4. Nine of these variables were represented by seven nutrients (crude proteins, total starch, cystine, methionine, total iron, total zinc, and spermidine) (Table 3) and two indices: in vitro raw protein digestibility and in vitro native starch digestion expressed as "native starch **h**ydrolysis **i**ndex" (HI) (Table 4). The nutrient values of two additional variables, namely, "cystine/crude proteins ratio" and "methionine/crude proteins ratio" (Table 4) were obtained by mathematic computation. In the same Table, the data and statistical analyses of two compounds exhibiting antinutritional effect (condensed tannins and total phenolics) and of ash, are also reported. Finally, the data and the results of statistical analyses of sapogenols (oleanolic acid, soyasapogenols A and B), hemicellulose, cellulose, lignin and trypsin inhibitors, are shown in Table 5. The data of 14 variables out of 19 examined fitted the preliminary conditions of normality and variance homogeneity (Table 2) and, therefore, they were submitted to ANOVA and Duncan's test. The data of five variables, namely, *spermidine*, *raw protein digestibility*, *native starch hydrolysis* (HI), *condensed tannins,* and *soya-sapogenol B* did not satisfy the above conditions (Table 2) and, therefore, the possible variation between lines was assessed by applying the non-parametric *Kruskal-Wallis test* (results reported in Table 2).

Table 2. Summary of the assessment of preliminary conditions for the applicability of the analysis of variance for all recorded variables on a set of thirteen common bean lines.

Variable	Normality of Variable Data (*Skewness and Kurtosis Test*) [a]		Homogeneity of Variance (*Bartlett's Test*) [c]	Normality of Residua (*Shapiro-Wilk Test*) [d]	ANOVA Results [e]	Evaluated Variation among Bean Lines (*Kruskal-Wallis Test*) [f]
	Raw Data = X	Data Transformation [b]				
Crude proteins	yes		yes	yes	$p < 0.001$	
Total starch	yes		yes	yes	$p < 0.001$	
Cystine	no	(1000/X)^1.3.	yes	Raw data = no **; Transf. data = yes	(Only transf. data) $p < 0.001$	
Methionine	yes		yes	yes	$p < 0.001$	
Total iron	yes		yes	yes	$p < 0.001$	
Total zinc	yes		yes	yes	$p < 0.001$	
Spermidine	no	(X^3.86)/(10^8)	no *	Both raw and transf. yes	–	31.81 **
Raw protein digestibility	no	(X^6)/(10^9)	yes	Raw data = yes Transf. data = no ***	–	22.87 *
Native starch hydrolysis index (HI)	no	(10^10)/(X^4.5)	yes	Raw data = no ***; Transf. data = no **	–	20.80 *
Cystine/crude prot.	no	(10^3)/X	yes	Both raw and transf. yes	$p < 0.001$	
Methion./crude prot.	yes		yes	yes	$p < 0.001$	
Condensed tannins	no	no	no *	yes	–	35.62 ***
Total phenolics	no	X^1.163	yes	Both raw and transf. yes	$p < 0.001$	
Oleanoic acid	no	(X^0.5) × 100	yes	Both raw and transf. yes	$p < 0.001$	
Soya-sapogenol A	no	(X^0.5) × 100	yes	Both raw and transf. yes	$p < 0.01$	
Soya-sapogenol B	yes		yes	no *	–	15.08 ns
Hemicellulose	no	1000/X	yes	Both raw and transf. yes	$p < 0.001$	
Cellulose	no	(10^5)/(X^1.75)	yes	Both raw and transf. yes	$p < 0.001$	
Lignin	yes		yes	yes	$p < 0.001$	

[a] yes: normal data according to both *skewness* and *kurtosis test*; [b] transformed data of all transformed variables were normal; the data of the variable *cond. tannins* could not be normalized; [c] yes: homogeneous variances (probability of *Bartlett's test* $p > 0.05$), *: *Bartlett's test* significant at $[0.01 < p \leq 0.05]$; [d] yes: normal residuals (probability of *Shapiro-Wilk* test $p > 0.05$), *, **, ***: *Shapiro-Wilk test* significant at $[0.01 < p \leq 0.05]$, $p \leq 0.01$ and $p \leq 0.001$, respectively; [e] *F* test probability of variation among lines from the ANOVA analysis; [f] non-parametric assessment of variation for the variables not complying with the preliminary conditions for the applicability of the ANOVA analysis, ns, *, **, ***: χ^2 value of the *Kruskall-Wallis test* not significant and significant at $[0.01 < p \leq 0.05]$, $p \leq 0.01$ and $p \leq 0.001$, respectively. (^) Excel sign meaning 'Raise to a determinate power'.

Table 3. Means and statistical results of seven nutritional variables surveyed in the flour of thirteen bean materials (the control cultivar BAT881 and twelve developed lines) having different genetic backgrounds. All values are expressed on a dry matter basis. The genotypic values of each variable were the means calculated between the replicated data.

Variable no. →		1		2		3		4		5		6		7
No.	**Line/cv**	**Crude Proteins (% ± SD)**		**Total Starch (% ± SD)**		**Cystine (mg g^{-1} ± SD)**		**Methionine (mg g^{-1} ± SD)**		**Total Iron (µg g^{-1} ± SD)**		**Total Zinc (µg g^{-1} ± SD)**		**Spermidine (µg g^{-1} ± SD) ***
1	HP5/1	26.48 ± 0.123	C	36.22 ± 0.788	F	7.80 ± 0.295	C	6.45 ± 0.243	B	74.74 ± 4.790	D	31.62 ± 2.026	E	764.8 ± 71.16
2	730/1	23.65 ± 0.124	F	37.16 ± 0.395	EF	8.55 ± 0.324	B	5.35 ± 0.204	G	75.01 ± 4.531	D	31.63 ± 1.911	E	878.3 ± 3.337
3	042/5	24.14 ± 0.239	F	38.05 ± 0.236	DEFG	7.30 ± 0.275	D	5.90 ± 0.223	D	91.37 ± 5.722	A	35.55 ± 2.226	B	770.4 ± 58.20
4	041/1	26.03 ± 0.036	CD	41.32 ± 0.630	AB	7.20 ± 0.271	D	5.75 ± 0.219	E	84.77 ± 4.991	B	37.94 ± 2.234	A	873.0 ± 42.94
5	720/12	23.63 ± 0.058	F	40.60 ± 1.027	ABC	7.20 ± 0.272	D	6.15 ± 0.232	C	83.77 ± 5.284	B	34.36 ± 2.167	C	735.5 ± 18.63
6	WH1/28	28.91 ± 0.485	A	36.35 ± 0.551	F	4.65 ± 0.175	G	6.75 ± 0.255	A	67.07 ± 4.060	F	31.27 ± 1.893	EF	506.7 ± 35.31
7	WH3/76	27.63 ± 0.327	B	37.62 ± 0.474	DEF	4.95 ± 0.188	F	6.45 ± 0.244	B	72.86 ± 4.387	E	33.55 ± 2.020	D	777.6 ± 6.274
8	WH4/84	23.94 ± 0.378	F	37.61 ± 1.027	DEF	4.95 ± 0.188	F	5.85 ± 0.220	E	66.57 ± 3.753	F	24.50 ± 1.381	I	835.0 ± 0.333
9	BR2/11	24.87 ± 0.131	E	40.21 ± 0.790	ABCD	4.95 ± 0.188	F	5.45 ± 0.208	F	63.26 ± 3.940	G	27.45 ± 1.710	G	734.6 ± 43.44
10	938	22.40 ± 0.282	G	42.09 ± 0.157	A	5.35 ± 0.203	E	5.45 ± 0.207	F	72.26 ± 4.589	E	26.23 ± 1.666	H	815.5 ± 32.15
11	720/18	25.65 ± 0.065	D	39.40 ± 0.395	BCDE	4.55 ± 0.172	G	5.85 ± 0.220	E	75.09 ± 4.639	D	34.65 ± 2.140	C	800.0 ± 27.95
12	720/20	25.33 ± 0.145	DE	38.61 ± 0.393	CDEF	15.0 ± 0.568	A	6.10 ± 0.231	C	71.59 ± 4.455	E	27.53 ± 1.713	G	746.5 ± 0.415
13	BAT 881	25.34 ± 0.058	DE	41.22 ± 1.575	AB	4.50 ± 0.171	G	5.45 ± 0.207	F	77.42 ± 4.822	C	30.65 ± 1.909	F	627.4 ± 38.98
No. of replications		2		2		2		2		2		2		3
Maximum value		28.91		42.09		15.00		6.75		91.37		37.94		878.3
Minimum value		22.40		36.22		4.50		5.35		63.26		24.50		506.7
Max/min		1.29		1.16		3.33		1.26		1.44		1.55		1.73
Coeff. of variation (%)		0.90		2.00		1.78		0.45		0.72		0.80		17.6

All values not sharing a common letter are significantly different at $p \leq 0.01$ (capital letters); * variation among lines was assessed according to the *Kruskal-Wallis* test (see Table 2).

Table 4. Means and statistical results of four nutritional and two antinutritional variables, and of ash, surveyed in the flour of thirteen bean materials (the control cultivar BAT881 and twelve developed lines) having different genetic backgrounds. All values are expressed on a dry matter basis. The genotypic values of each variable were the means calculated between the replicated data.

Variable No. →		8	9	10		11		12	13		14
No.	**Line/cv**	**Raw Protein Digestibility (% ± SD) [a]**	**Native Starch Hydrolysis Index (HI ± SD) [b]**	**Cystine/Crude Proteins (% ± SD)**		**Methionine/Crude Proteins (% ± SD)**		**Cond. Tannins (mg of Delphinidin Equiv. g^{-1} ± SD) [c]**	**Total Phenolics (mg of Gallic Acid Equiv. g^{-1} ± SD)**		**Ash (%)**
1	HP5/1	79.99 ± 0.824	52.77 ± 0.824	2.94 ± 0.125	CD	2.43 ± 0.103	B	7.507 ± 0.293	15.540 ± 0.610	A	5.08
2	730/1	82.03 ± 0.740	50.70 ± 2.482	3.62 ± 0.156	B	2.28 ± 0.098	CD	6.680 ± 0.310	13.947 ± 0.550	B	5.36
3	042/5	80.08 ± 0.821	48.33 ± 2.528	3.01 ± 0.084	C	2.44 ± 0.068	B	6.917 ± 0.260	12.800 ± 0.511	C	4.85
4	041/1	77.11 ± 0.943	42.25 ± 2.832	2.76 ± 0.108	D	2.22 ± 0.087	DEF	4.737 ± 0.344	8.037 ± 0.182	E	4.62
5	720/12	78.04 ± 0.905	41.83 ± 2.341	3.04 ± 0.108	C	2.59 ± 0.092	A	6.613 ± 0.261	11.550 ± 0.201	D	5.07
6	WH1/28	79.05 ± 0.863	48.02 ± 3.073	1.60 ± 0.088	H	2.33 ± 0.127	C	0.010 ± 0.000	3.497 ± 0.303	F	4.68
7	WH3/76	83.06 ± 0.698	50.03 ± 3.533	1.79 ± 0.089	G	2.33 ± 0.116	C	0.010 ± 0.000	3.497 ± 0.390	F	4.38
8	WH4/84	79.97 ± 0.825	43.46 ± 1.924	2.07 ± 0.111	F	2.43 ± 0.130	B	0.010 ± 0.000	3.723 ± 0.087	F	4.20
9	BR2/11	77.43 ± 0.930	43.55 ± 2.604	1.99 ± 0.086	F	2.21 ± 0.095	EF	6.740 ± 0.209	13.383 ± 0.323	BC	4.22
10	938	80.10 ± 0.820	42.70 ± 1.123	2.39 ± 0.060	E	2.44 ± 0.062	B	6.283 ± 0.269	13.680 ± 0.256	B	4.69
11	720/18	83.18 ± 0.693	48.78 ± 2.827	1.77 ± 0.072	G	2.27 ± 0.092	CDE	3.677 ± 0.081	11.080 ± 0.352	D	4.96
12	720/20	80.67 ± 0.796	48.23 ± 3.513	5.92 ± 0.191	A	2.40 ± 0.077	B	3.860 ± 0.270	10.863 ± 0.381	D	4.69
13	BAT 881	71.75 ± 1.164	42.25 ± 2.780	1.79 ± 0.072	G	2.16 ± 0.087	F	6.563 ± 0.272	13.133 ± 0.214	BC	5.16
No. of replications		2	2	2		2		3	3		1
Maximum value		83.18	52.77	5.92		2.59		7.507	15.54		5.36
Minimum value		71.75	41.83	1.60		2.16		0.010	3.497		4.20
Max/min		1.16	1.26	3.68		1.20		750.7	4.44		1.28
Coeff. of variation (%)		0.34	5.82	2.00		0.88		6.62	5.31		

All values not sharing a common letter are significantly different at $p \leq 0.01$ (capital letters); [a] Percent of crude protein digested in vitro in 180 min; [a], [b], [c]: Variation among lines was assessed according to the *Kruskal-Wallis* test (see Table 2).

Table 5. Means and statistical results of seven antinutritional variables surveyed in the flour of thirteen bean materials (the control cultivar BAT881 and twelve developed lines) having different genetic backgrounds. All values are expressed on a dry matter basis. The genotypic values of each variable were the means calculated between the replicated data.

Variable No. →		15		16		17	18				19		20	21
No.	**Line/cv**	**Sapogenols**					**Hemicellulose (% ± SD)**		**Cellulose (% ± SD)**		**Lignin (ADL) (% ± SD)**			**Trypsin Inhibitors (µg mg^{-1})**
		Oleanolic Acid (µg g^{-1} ± SD)		**Soya-Sapogenol A (µg g^{-1} ± SD)**		**Soya-sap. B (µg g^{-1} ± SD) ***								
1	HP5/1	245 ± 5.962	A	30 ± 0.369	BCD	1060 ± 14.14	20.72 ± 0.861	EFG	7.330 ± 0.301	D	1.500 ± 0.276	CDEF		8.27
2	730/1	135 ± 21.21	BC	65 ± 7.071	ABC	1525 ± 49.50	20.99 ± 0.582	EF	6.993 ± 0.303	DE	2.350 ± 0.077	AB		15.72
3	042/5	100 ± 14.14	CD	60 ± 14.14	ABC	1510 ± 70.71	27.76 ± 0.195	B	7.727 ± 0.275	CD	2.093 ± 0.033	ABC		6.95
4	041/1	120 ± 12.10	BCD	30 ± 0.527	BCD	1350 ± 482.5	19.63 ± 0.607	GH	6.147 ± 0.230	F	2.027 ± 0.196	ABCD		10.40
5	720/12	105 ± 8.300	CD	40 ± 25.57	ABCD	1355 ± 177.8	25.74 ± 0.594	C	6.567 ± 0.373	EF	2.260 ± 0.191	AB		7.05
6	WH1/28	145 ± 22.43	BC	30 ± 16.41	BCD	1120 ± 141.2	22.46 ± 0.468	D	9.807 ± 0.111	AB	1.313 ± 0.170	EF		3.19
7	WH3/76	30 ± 14.14	F	30 ± 14.14	BCD	990 ± 14.14	20.31 ± 0.327	FG	9.607 ± 0.355	AB	0.910 ± 0.264	F		6.20
8	WH4/84	55 ± 18.81	EF	100 ± 14.14	A	1430 ± 84.85	30.94 ± 0.925	A	10.36 ± 0.199	A	1.550 ± 0.195	CDEF		6.94
9	BR2/11	180 ± 28.28	AB	80 ± 14.14	AB	1400 ± 548.4	26.23 ± 0.438	BC	8.627 ± 0.316	BC	2.533 ± 0.435	A		7.60
10	938	120 ± 14.14	BCD	65 ± 34.90	ABC	1160 ± 70.71	21.77 ± 0.610	DE	8.413 ± 0.373	BC	1.383 ± 0.291	DEF		7.51
11	720/18	55 ± 7.071	EF	15 ± 6.823	D	1225 ± 20.49	22.29 ± 0.905	D	9.227 ± 0.306	AB	1.750 ± 0.216	BCDE		5.32
12	720/20	75 ± 7.189	DE	25 ± 7.922	CD	1375 ± 145.3	19.13 ± 0.381	HI	7.000 ± 0.666	DE	2.333 ± 0.479	AB		3.14
13	BAT 881	35 ± 8.640	F	25 ± 7.071	CD	1160 ± 56.57	18.50 ± 0.101	I	7.633 ± 0.264	CD	2.417 ± 0.120	AB		6.56
No. of replications		2		2		2	3		3		3			1
Maximum value		245		100		1525	30.94		10.36		2.533			15.72
Minimum value		30		15		990	18.50		6.147		0.910			3.14
Max/min		8.17		6.67		1.54	1.67		1.69		2.78			5.01
Coeff. of variation (%)		7.66		17.4		17.9	2.51		4.19		14.2			

All values not sharing a common letter are significantly different at $p \leq 0.01$ (capital letters); * Variation among lines was assessed by applying the *Kruskal-Wallis* test (see Table 2).

The ratio between maximum and minimum values (max/min) allowed to rapidly identifying the variables showing large differences between genotypic means. Condensed tannins showed the highest difference (max/min = 750.7) due to the presence of the *wsc* mutation which prevents the accumulation of these compounds in the seed coat. Following were total phenolics, oleanolic acid, soya-sapogenol A, trypsin inhibitors with ratio values between 8.17 and 4.44 (Tables 4 and 5). Intermediate max/min values were observed for the ratio cystine/crude proteins and the content of cystine and lignin (values ranging from 3.68 and 2.78), whereas low values (< 1.8) were observed for all other variables (Tables 3–5). The max/min values related to the content of methionine (1.26), total iron (1.44), total zinc (1.55) and spermidine (1.73) deserve particular attention due to the importance of these nutrients. Finally, the in vitro native starch digestion potentials expressed as hydrolysis index (HI), showed a max/min value of 1.26.

3.4. Results from the "First Group of Correlation Analyses"

These results are shown in Table 6. Out of 210 correlation coefficients computed, 19 resulted significant ($0.01 < p \leq 0.05$) and 8 highly significant ($p \leq 0.01$). Based on the type of compound correlated (nutrient or antinutrient), we identified five groups of correlations: (1) antinutrients vs. antinutrients, (2) nutrients vs. antinutrients, (3) nutrients vs. nutrients, (4) cellulose vs. (three nutrients + three antinutrients), (5) total starch and raw protein digestibility vs. in vitro native starch digestion. Four antinutrient compounds (lignin, trypsin inhibitors, sapogenols A and B) correlated negatively ($0.01 < p \leq 0.05$) vs. three main nutrients (methionine, total zinc and crude proteins). Curiously, cellulose correlated negatively vs. the main antinutrients (condensed tannins, lignin and total phenolics) but also vs. two nutrients (iron and cystine) as well as vs. ash.

Table 6. Results obtained from the *First group of correlation analyses* (see M&m). Significant and highly significant indices obtained by correlating each other the 21 genotypic variables reported in Tables 3–5 whose data were surveyed in 13 bean materials (the control cultivar BAT881 and twelve developed lines). For each variable, correlated values were 13 means each one calculated between the replicated data.

Variables A	Variables B	Correlation Value Variables A vs. B
Antinutrients vs. antinutrients		
Cond. tannins	Total Phenolics	+0.956 **
SoyasapogenolA	Hemicellulose	+0.751 **
Lignin	SoyasapogenolB	+0.691 **
"	Cond. tannins	+0.635 *
"	Total Phenolics	+0.574 *
Nutrients vs. antinutrients		
Spermidine	Trypsin inhibitors	+0.633 *
Ash	Total Phenolics	+0.594 *
"	Cond. tannins	+0.572 *
Methionine	Lignin	−0.591 *
"	Trypsin inhibitors	−0.550 *
Total zinc	SoyasapogenolA	−0.577 *
Crude proteins	SoyasapogenolA	−0.584 *
"	SoyasapogenolB	−0.593 *
Nutrients vs. nutrients		
Total starch	Methionine	−0.610 *
Crude proteins	Spermidine	−0.561 *
"	Methionine	+0.734 **
Total Fe	Total Zn	+0.759 **
Cystine	Cystine/Crude proteins	+0.991 **

Table 6. *Cont.*

Variables A	Variables B	Correlation Value Variables A vs. B
	Cellulose	
Cellulose	Cond. tannins	−0.727 **
"	Total Fe	−0.623 *
"	Lignin	−0.617 *
"	Total Phenolics	−0.606 *
"	Cystine/Crude proteins	−0.605 *
"	Cystine	−0.581 *
"	Ash	−0.574 *
	Other correlations	
Raw protein digestibility	Starch hydrolysis index (HI)	+0.654 *
Total starch	""""	−0.814 **

**: Significant at $p \leq 0.01$; *: Significant at $p = [0.01 < p \leq 0.05]$.

3.5. Results from the "Second Group of Correlation Analyses"

These results are shown in Table 7. Out of 84 correlation indices produced, nine resulted significant ($0.01 < p \leq 0.05$) and four (three vs. iron and one vs. cellulose) highly significant ($p \leq 0.01$).

Table 7. Results obtained from the *Second group of correlation analyses.* Significant and highly significant correlation indices showing the effect generated by three genetic traits (*Presence of the* lpa *mutation*, *Absence of phaseolin*, *Presence of the α-AI*) and by a combination of two of them (*Absence of phaseolin + Presence of the α-AI*) on the content of some nutrients/antinutrients in the seeds of the analyzed beans (see M&m and Table 1).

Genetic Condition/Variable	Correlation Value
Presence of the* lpa *mutation [a]	
Total Fe	− 0.645 *
Ash	−0.582 *
Total starch	−0.564 *
Cellulose	+0.637 *
Absence of phaseolin	
Total Fe	+0.719 **
Trypsin inhibitors	+0.615 *
Total Zn	+0.604 *
Cond. tannins	+0.548 *
Cellulose	−0.712 **
Presence of the α-AI	
Total Fe	+0.687 **
Absence of phaseolin + Presence of the α-AI [b]	
Total Fe	+0.839 **
Total Zn	+0.667 *
Cellulose	−0.553 *

** = Significant at $p \leq 0.01$; * = Significant at p $[0.01 < p \leq 0.05]$.

[a] The presence of the genetic mutation *lpa* in common bean, reduces to 10% the amount of PAP (**P**phytic **A**cid **P**hosphorus) in the seed; [b] Cumulative effect of the two traits produced when combined in the same genetic group of lines; **α-AI** = Alpha-Amylase Inhibitors.

4. Discussion

4.1. Nutrients and Antinutrients (a General View)

The values of the spermidine content were in general rather high (from 507 to 878 μg g^{-1} of dry matter; Table 3), especially in two lines (**730/1** = 878.25 μg g^{-1}; **041/1** = 873.01 μg g^{-1}). It is important to remark that such levels are close to those found in foods rich in this compound [59]. Notably, spermidine exerts its positive function in cardio-protection and lifespan extension [60]. Moreover, previous data reported on common bean, confirmed the presence of a relationship between polyamines concentration and abiotic stress tolerance [61]. In this work, the interrelations of spermidine with other compounds did not appear so sharp: it correlated significantly only vs. trypsin inhibitors (+0.633 *, $p = 0.019$) and vs. crude proteins (−0.561 *, $p = 0.044$; Table 6).

Results from this work might guide novel studies in common bean breeding aimed to raise the level of this polyamine accumulated in the seeds. In addition, the present findings, together with those previously published by Soda et al. (2009) [62], could help to deepen the knowledge on a possible role of polyamines in the food, in particular in that used in the Mediterranean diet, where these substances are generally abundant and are known to prolong the human life.

The in vitro native starch HI is an important nutritional indicator of the metabolic glycemic response. It is well recognized that the values of this variable are lower in legume seeds than those observed in cereals, due to the interplay of several factors related to the starch source, starch granule size and surface, type of crystalline polymorphic form, presence of protein and/or fiber matrices enclosing the starch granules, amylose/amylopectin ratio, as well as the presence of antinutrients [63]. The variation observed in the HI values of our genetic materials was narrow (max/min = 1.26), leaving little space to the use in breeding programs of the lines showing extreme values. The same consideration can be made for the in vitro protein digestibility, whose values fell between 71.75 and 83.18% of raw protein digested in 180 min (max/min = 1.16; Table 4).

4.2. All Correlations Analyses (First and Second Group)

Out of the forty correlations reported in Table 6; Table 7, twenty–seven were evaluated for the first time in this work, whereas thirteen were also analyzed in a previous work [40]. Out of the latter: (a) six were significant or highly significant in both works and maintained the same trend, confirming previous results (*condensed tannins/total phenolics, total Fe/total Zn, ash/lpa trait*, and *cellulose vs condensed tannins, total phenolics and lignin*); (b) seven were significant in this work but not in the previous one (*cellulose/ash, total phenolics/ash, condensed tannins/ash, lignin/condensed tannins, lignin/total phenolics, lpa trait/total Fe, cellulose/lpa trait*); (c) two were significant in the previous work but not in this one (*crude proteins/condensed tannins* and *crude proteins/total phenolics*); and (d) one was significant in both works but, with inverted trend (*Cellulose/Total Fe*; Table 6). The differences found in "b", "c" and "d" might be related to the presence of a higher genetic diversity in the materials analyzed in this work (Table 1) than in those analyzed in Campion et al. (2013) [40] which had a very similar genetic characteristics except for the trait analyzed.

The correlation lpa *mutation/total Fe seed content* obtained in this work (Table 7), unexpectedly produced a negative index value (−0.645 *, $p = 0.016$). This is not an encouraging result for breeders who plan to increase the iron content in *lpa* bean seeds. However, no differences were detected between *lpa* and *wt* beans for Fe content in Campion et al. (2013) [40], where the Fe analyses were performed only in lines producing phaseolin-containing seeds (but also genetically more uniform).

Finally, the correlation between *raw protein* in vitro *digestibility* vs *native starch hydrolysis index* (HI) was significant and positive (+0.654 *, $p = 0.014$, Table 6]. Present results suggest that the digestion process can be considered as a cooperative process, in which a greater hydrolysis of one substrate may be affected by the simultaneous hydrolysis of another. In particular, since the protein matrix in legume grains can hinder the native starch hydrolysis during digestion, a greater raw protein in vitro digestibility may contribute to facilitate the access of starch degrading enzymes.

4.3. Interrelations vs the "Absence of Phaseolin" and vs the "Presence of the α-AI"

The genetic elimination of phaseolin, the major protein accumulated in the common bean seeds, was previously described [24,25] and recently elucidated at molecular level [64]. One of the aim of the first researches was to raise the amount of methionine by modifying the equilibrium of the type of proteins accumulated in the bean seed. In this work, we verified whether the removal of this main storage protein could induce unkown changes in the content of nutrients and/or antinutrients in the bean seed.

By observing the results obtained from the correlations performed between the four theoretical variables vs. the quantitative ones, we firmly realized that, *theoretical variables* vs *total iron*, *total zinc* and *cellulose*, were repeatedly significant (Table 7). The effect produced singly by the *Absence of phaseolin*, and that produced by the *Presence of the α-AI*, was highly significant for both *vs total iron content* (+0.719 ** and +0.687 **, respectively). In addition, the combination of both traits (*Absence of phaseolin* + *Presence of the α-AI*) vs *total iron content*, produced the highest correlation value (+0.839 **), due to a cumulative effect of the two traits when combined in the same genetic group (lines 042/5, 041/1 and 720/12). A similar response, but with slighter effect, was also observed for *total zinc* content, produced by the *Absence of phaseolin* (+0.604 *) and by the combination of the two traits, *Absence of phaseolin* + *Presence of the α-AI* (+0.667 * at a $p = 0.012$; Table 7). Concerning this last correlation, it is noteworthy that its lightly higher value than the former one suggests an additional positive effect produced by the *Presence of the α-AI*. The similarity of response obtained here between iron and zinc was in part expected, since iron and zinc are in general highly correlated {(+ 0.759 ** in this work; Table 6) and [40,65]}. It was also evident the reduction of the cellulose content, which was high in the condition of *Absence of phaseolin* (−0.712 **) but slightly lower when this genetic trait was in combination with the presence of the *α-AI* (−0.553 *, $p = 0.048$; Table 7).

Finally, the seeds of three lines (042/5, 041/1, and 720/12) carrying the genetic combination *Phaseolin absence* + *α-AI presence* (Table 1), showed a higher content of iron (+ 22.4%) than the baseline (which is 70.76 μg g^{-1}; mean calculated between the iron content of normal phaseolin containing lines). In particular, the seeds of one of them, namely, the line 042/5, contained 29.1% more iron than the baseline (91.37 μg g^{-1}; Table 3). It is important to remark that the high levels of iron (and, in part of zinc as well), recorded in the seeds of the bean lines 041/1, 042/5 and 720/12, were achieved without any specific breeding intervention.

Another correlation that deserves consideration is *phaseolin absence* vs *protein digestibility*. It is well-known that phaseolins, especially those types present in most bean cultivars, are marginally digested in vitro and in vivo, mostly due to their resistance to hydrolysis and/or proteolysis [26]. On the other hand, it has been demonstrated that also other proteins, that are accumulated in the common bean seed besides phaseolin (legumin, albumin, glutelin and prolamin), are little digested [66,67]. The null correlation value obtained in the present work (−0.008 ns; not reported in Tables) showed the absence of any effect and could be considered in line with what previously reported by the above Authors.

4.4. "Phaseolin Absence + lpa Mutation", an Inedited Genetic Combination

Petry et al. (2016) [68] demonstrated that the presence of the *lpa* mutation carried by the bean 586/8X87-brown (provided by CREA-GB) could improve the total Fe absorption per test meal in Rwandese women with low iron status at the same level of the biofortified bean (provided by CIAT) which was used in the experiment. In particular, the results showed that the total amount of iron absorbed from *lpa* beans and the one absorbed from biofortified beans did not differ significantly, whereas both were >50% higher ($p < 0.005$) than the iron amount absorbed from the bean landrace G4825 used as the control. For this reason, the *lpa* bean line 586/8X87-brown was used as the female parent for the development of the new bean line HP5/1, carrying an inedited genetic trait combination: *lf* + *lpa* + *absence of phaseolin* (Table 1). HP5/1 was developed with the objective to associate the positive effect of the *lpa* mutation on Fe absorption described by Petry et al. (2016) [68], together with that of a higher Fe accumulation in the seed (described in the present work) induced by the *Absence of*

phaseolin. The target idea was to maximize the amount of total bioavailable Fe absorbed from the meal by the human body. Unfortunately, this new *lpa* bean, together with other six ones listed in Table 1, contains variable amounts of the phytohaemagglutinin PHA-L, which seems to give serious health problems to humans when combined with *lpa* mutation [68]. In fact, the consumption of the *lpa* bean (586/8X87-brown) during the experiment carried out in Rwanda caused transient adverse digestive side effects in 95% of participants [68]. A gel electrophoresis analysis performed later, showed the presence of phytohaemagglutinin L-type (PHA-L), not only in the raw flour, but also in the residues of the cooked *lpa* bean [69]. In a recent work, the interaction between PHAs (E and L) and the *lpa* trait was investigated, and the Authors demonstated that the *lpa*1 mutation in combination with the presence of different PHA alleles, affected the stability of the PHA-L lectin, due to an excess of free cations (Cominelli et al. unpublished). It is important to note that, besides the poisonous lectin PHA-E missing in all bean lines except the control cv BAT 881, the other missing seed lectin proteins in all *lpa* beans reported in Table 1, were *α-AI* and *Arcelin* (PHA-L was still present). Based on the above published results, possible introgressions of the trait responsible of the accumulation of PHA-L should be avoided in a future bean breeding work aimed to associate lpa *mutation* and *absence of phaseolin* in the same genetic background.

Concerning the nutritional seed composition, HP5/1 did not present substantial differences from normal beans. Only the oleanolic acid (one of the three sapogenols analyzed) was present at the highest level compared to the other genotypes, with a seven-times higher level than the control value (Table 5).

4.5. "Phaseolin Absence + α-AI Presence", a Possible Tool to Increase Iron and Zinc in the Bean Seed?

The high correlation between "*Phaseolin absence + α-AI presence*" vs "i*ron and zinc* content" in the bean seeds, is the second unprecedented result obtained in the present work. It certainly deals with one of the most important topic of food research worldwide.

Iron is an essential micronutrient for almost all living organisms [70], and its deficiency is considered as one of the most common micronutrient deficiency worldwide [15,71–74]. Iron deficiency is difficult to meet through Fe supplementation or processed foods, and several tools, in particular seed biofortification, are required, therefore, to enhance Fe accumulation into staples such as rice, maize, wheat, and legumes [75,76]. In *P. vulgaris*, the need to raise the Fe seed content over 105 μg g^{-1} is the focus of many bean researchers who want to improve further the Fe absorption at intestinal level of mammalians [77].

Blair et al. (2009) [78] identified a total of 26 QTL of which half were for iron concentration and half for zinc concentration, suggesting that the accumulation of iron and zinc in the common bean seed is controlled multigenically. The same Authors also reported a first evidence of the association "*Phaseolin / iron + zinc accumulation*" where common QTL for the two minerals were found on linkage group B7 in the interval M125D to A143G, and where the phaseolin seed protein locus is located.

In the present work, the correlation values between the two genetic traits "*Absence of phaseolin*" and "*Presence of the α-AI*", singly or combined vs. the "*Amount of iron accumulated in the seeds*", resulted all positive and very high (+0.719 **, +0.687 ** and +0.839 **, respectively; Table 7). In particular, the level of iron recorded in the seeds of the line 042/5 (91.37 μg g^{-1}) was obtained without any specific breeding intervention. Considering the QTL analyses described by Blair et al. (2009) [78] together with our results, we might put forward two hypotheses to explain the relatively higher amount of iron and zinc accumulated in the lines 042/5, 041/1 and 720/12. The first hypothesis is that the result might derive only from a casual association of useful genetic traits obtained from the particular crosses made and the selection methods applied. According to the second hypothesis, a real genetic/metabolic interaction would act between the involved genetic traits "*Phaseolin absence + α-AI presence*" vs "*Iron and zinc accumulation*", revealing the presence of a powerful and important influence of the first two traits in raising the amount of the two minerals in the bean seeds. The results obtained in the present work might be a first indication of the phenomenon, supporting the second hypothesis. Unfortunately, so far we have not complete proofs to confirm its full reliability, due to the presence of a rather high genetic

variability between the twelve bean lines, even though, on the other hand, very high correlations were observed, firmly suggesting to look for a scientific confirmation. For instance, a further verification carried out on "near isogenic lines" could give a definitive answer to this question.

5. Conclusions

Based on our results, and waiting for confirmation by further scientific verification, the combined presence of the genetic traits *Absence of phaseolin* + *Presence of α-AI* in a bean genetic background, might be potentially determinant for raising iron and zinc seed content in the framework of a biofortification breeding program. Moreover, an additional introgression of the *lpa* mutation in the above genetic combination, might allow improving further the Fe absorption at the intestinal level in humans.

Author Contributions: *G.G.*: Chemical analyses of nutrients (except spermidine); raw protein digestibility and native starch hydrolysis (HI) evaluation; manuscript revision. *A.T.*: Chemical analyses of antinutrients (except trypsin inhibitors); dry matter and ash determination; manuscript revision. *G.M.*: Chemical analyses for spermidine determination; manuscript revision. *L.P.*: Verification of the conditions of applicability of ANOVA trough the: (1) application of the *Shapiro-Wilk* test to evaluate the normality of residuals; (2) evaluation of the variance homogeneity by applying the *Bartlett test* to the data of all variables (raw and transformed). Evaluation of the genetic variation among lines in the non-normally distributed variables by applying the non-parametric *Kruskal-Wallis* test. Manuscript revision. *F.M.*: Critical review of the manuscript, in particular towards the results obtained from chemical analyses of the nutrients. *F.S.*: Chemical analyses of trypsin inhibitors; analyses of protein profile of a few bean lines; manuscript revision. *A.L.F.*: Cultivation of the bean lines and cv BAT881 according to the organic farming system for the production of the seed material. *B.C.*: Production of the genetic materials and co-ordination of the whole research. Accomplishment of part of the statistical analyses applied to the data (normalization by applying the skewness and kurtosis test, ANOVA analysis, Duncan's range test, computation of simple correlations). Manuscript drafting and revision.

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Appendix A

Table A1. Main steps of development and other information related to the bean lines/cv listed in Table 1, evaluated for seed nutritional conposition.

Schematic pathway showing the origin of the lines **720/18** and **720/20** **1st step:** (♀Taylor's Horticultural × Pinto UI 111♂) → Lady Joy [(Pinto bean lectin + α-AI) borlotto line] (Roberto Bollini and Bruno Campion, unpublished); **2nd step:** [♀BAT 881 × Lady Joy♂] → lines **720/18** and **720/20**.
Schematic pathway showing the origin of the lines **041/1**, **042/5** and **720/12** (Bruno Campion and Roberto Bollini, unpublished) **1st step:** [♀Lady Joy x selected phaseolin-free plants of Venere♂] → biochemical and agronomic selection → **P22** (F_2 plant); **2nd step:** (♀938 × P22 F_2 plant♂→ biochemical and agronomic selection → intercrosses between breeding lines → lines **041/1**, **042/5**, and **720/12**. –Venere = white seeded *P. coccineus* L. cultivar exhibiting determinate growth habit [79]. –938 genetic background = ♀BAT881 × (♀A55 × G6388♂)♂. The origin of the line 938 is described in Campion et al. (2009 and 2013) [37,40].
Schematic pathway showing the origin of the lines **730/1** (Bruno Campion and Roberto Bollini, unpublished), **WH4/84** and **HP5/1** **1st step:** [♀Lady Joy x selected phaseolin-free plants of Venere♂] → biochemical and agronomic selection → **P22** (F_2 plant); **2nd step/b:** P22 (F_2 plant) → P22 (F_4 plants) → [♀938 × P22 (F_4 plants)♂] → biochemical / agronomic selection → line **730** → agronomic selection → line **730/1**. **3rd step/a:** (♀*lpa* line 586/8X87-white x 730♂) → biochemical and agronomic selection → line **WH4/84**. **3rd step/b:** (♀*lpa* line 586/8X87-brown x 730♂) → biochemical and agronomic selection → line **HP5/1**. –586/8X87-brown and 586/8X87-white = *lpa* lines developed at CREA-GB [40].
Schematic pathway showing the origin of the lines **WH1/28**, **WH3/76** and **BR2/11** **1st step/a:** *lpa* line 586/8X87-white → biochemical and agronomic selection → lines **WH1/28** and **WH3/76**. **1st step/b:** *lpa* line 586/8X87-brown → biochemical and agronomic selection → line **BR2/11**.
The development of the lines 730/1, 720/18, 720/20, 042/5, 041/1 and 720/12, was performed at CREA-GB Research Centre of Montanaso Lombardo–Italy (Bruno Campion, unpublished) in two steps: **1st step)** crosses and selection analyses, made in the years 2001–2005 in collaboration with Dr. Roberto Bollini at IBBA-CNR of Milan (Istituto di Biologia e Biotecnologia Agraria–IBBA-CNR, Via Bassini 15–20133 Milano, Italy); **2nd step)** additional agronomic selection and evaluation, made in the years 2006–2015. The lines WH4/84 and HP5/1 were both developed in the years 2009–2015 at CREA-GB Research Centre. The cv BAT 881, used as the control, is a CIAT accession kindly provided by Prof. Shree Singh (CIAT = Centro Internacional de Agricultura Tropical, Cali, Colombia, see also CGIAR (www.cgiar.org).

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