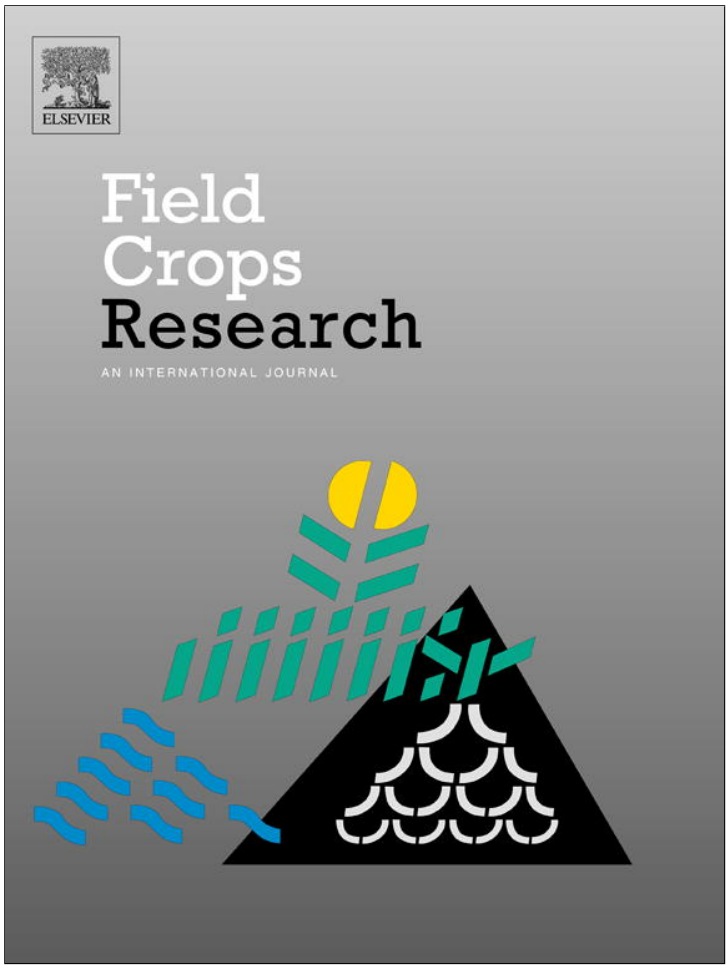

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Genetic reduction of antinutrients in common bean (*Phaseolus vulgaris* L.) seed, increases nutrients and *in vitro* iron bioavailability without depressing main agronomic traits

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ABSTRACT

In common bean, lectins, phytic acid, polyphenols and tannins exert major antinutritional effects when grains are consumed as a staple food. Reduced iron and zinc absorption, low protein digestibility and high toxicity at the intestinal level are the causes of their antinutritional effect. To improve grain nutritional characteristics, the "low phytic acid" (*lpa*) trait recently obtained in bean and carried by the bean mutant *lpa*-280-10, was introgressed into different lectin-free (*lf*) lines, a few of which were white-seeded. The "*white seed coat*" (*wsc*) trait is correlated with a reduced amount of tannins and polyphenols in bean seed, and thus higher Fe bioavailability. *Lf+lpa* bean lines producing colored and white seeds, were developed. Three of these lines were submitted to a first field performance test carried out in two Italian locations, and two of them to biochemical analyses that evaluated fourteen nutritional parameters. Seedling emergence and grain yield of *lf+lpa* beans were statistically comparable to those of wild type cultivars, confirming the absence of major agronomic defects associated with the *lpa* trait. The presence of the three genetic traits *lf*, *lpa* and *wsc* in the same genetic background leads to a significant increase of the content of important nutrients such as crude proteins, total zinc, free phosphorus, and, in part, total iron. Iron bioavailability (as measured *in vitro via* a Caco-2 cell model) in *lf+lpa* brown and black seeds, was not significantly different from that surveyed in the wild type colored parents, while, it was on average twelve times higher in *lf+lpa* white bean seeds. Up to now, the white-seeded *lf+lpa* beans seem thus to be the only materials having really improved nutritional qualities.

1. Introduction

Bean grains contain some substances endowed with biological activity that can be beneficial for health or exert serious antinutritional effects. The most important of these effects (*i.e.* reduction of iron and zinc bioavailability/absorption at intestinal level, low protein digestibility) are produced by polyphenols and tannins as well as phytic acid, and by protease inhibitors and lectins, especially phytohaemagglutinin (PHA). Because of their toxic effect, these proteins prevent the use of raw beans for monogastric animal feeding (Banwell et al., 1983; Greer and Pusztai, 1985). The presence of genetic materials with PHA-free seeds is rather common in *Phaseolus vulgaris* L. (Staswick and Chrispeels, 1984) whereas the development of bean lines able to produce completely lectin-free seeds, is a relatively recent achievement (Campion et al., 2009a).

In well-fed populations eating a diverse diet including meat, the health benefits afforded by the above compounds may be regarded as more important than the antinutritional effects. In this view,

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polyphenols are active against renal calculi (Grases et al., 2007) and, against the onset of cancer (Qu et al., 2005; Singh and Agarwal, 2005; Vucenik and Shamsuddin, 2006), condensed and hydrolyzable tannins are effective antioxidants (Rice-Evans et al., 1997; Hagerman et al., 1998), saponins are anticholesterol and antimutagenic agents (Berhow et al., 2000; Mittal and Jiang, 2004). On the other hand, when bean seed is utilized as staple food (like by African and Latin American people and by vegetarians), the antinutritional effect caused by these compounds prevails, leading to a decrease in feed intake and growth rate (Elías et al., 1979; Griffiths and Moseley, 1980; Bender and Reaidi, 1982; Bressani et al., 1982; Aw and Swanson, 1985; Reddy et al., 1985; Tako and Glahn, 2011).

Iron deficiency is still considered as the most widespread micronutrient deficiency world-wide (Lynch, 2011; Stoltzfus, 2011) and it has been considered the sixth highest cause of mortality in the world (WHO document 1992; The World Health Report, 2002). In the fight against iron malnutrition, the fundamental approaches lie in increasing the iron content of cereals and legumes (Welch et al., 2000; Welch, 2002) and/or improving its absorption by decreasing the presence of phytic acid, polyphenols and tannins, and lectins (Raboy, 2001; Welch and Graham, 2004). The latter strategy may lead to improve protein digestibility as well (Bressani et al., 1982; Norton et al., 1985).

Concerning iron and zinc deficiency, CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia; see also CGIAR – www.cgiar.org) targeted an important research activity for improving the bioavailability of these two cations in common bean (HarvestPlus Challenge Program – Biofortified Beans – CIAT Annual Report, 2006–2007). The developed biofortified bean lines are able to accumulate high levels of iron and zinc in the seeds but, how much of this iron is bioavailable, probably depends on the type of consumer studied (animal or human being) since published data appear at the moment not decisive. Tan et al. (2008) in piglets and Tako et al. (2011) in poultries, state that the above biofortified beans can provide superior amounts of bioavailable iron than normal beans while, Petry et al. (2012) in humans, show the iron of biofortified beans is not more bioavailable than that of normal ones concluding that "the common bean has limited potential as a vehicle for iron biofortification".

Raising the nutritional quality of common bean grain was also the main goal of our breeding programs following the route of reduction/elimination of antinutrients (Raboy, 2001; Welch and Graham, 2004). We isolated and characterized a new *lpa* (low phytic acid) mutant (*lpa*-280-10) with a reduced amount of phytic acid (90% reduction) and absence of lectins (*lf*) in the seeds (Campion et al., 2009b; Panzeri et al., 2011). Through further breeding activity we combined the *lf* + *lpa* traits with the *wsc* (white seed coat) trait in the same genetic background (Campion et al., 2009b; Perrone et al., 2009), to produce additive, combined effects. Since the *wsc* trait is associated with a low content of polyphenols and tannins in the bean seed (Ma and Bliss, 1978; Reddy et al., 1985), the new breeding lines might theoretically be considered "*biofortified*". In the present work we verify the reliability of this hypothesis (first goal), and report striking results showing higher *in vitro* iron bioavailability (using the Caco-2 cells system) and improved nutritional composition and quality of the new *lf* + *lpa* bean lines. Since different authors have observed in all species so far studied, that *lpa* mutations are associated with plant defects such as stunted vegetative growth, impaired seed germination capacity and seed emergence delay, reduced seed yield (Raboy et al., 2000; Pilu et al., 2005; Guttieri et al., 2006; Bregitzer and Raboy, 2006), the second goal of this work, not less important, was to gather additional data on the field behavior of our *lpa* beans. In Campion et al. (2009b), we conjectured for the first time the absence of major plant defects in *lpa* beans, based on the observed unexpected high plant vigor and grain yield of our *lpa* 280/10 mutant. In that work we also examined

Table 1
Phenotypic and biochemical traits of plant materials tested for field performance and, of a few of them, for biochemical evaluation.

Traits	Cv-line	Teggia	Sorano	BAT881[a]	A55[a]	938[a]	586/8[a]	586/8X61[b]	586/8X87[a,b]	586/8X147[a,b]
Plant growth habit[d]		det. Ia	det. Ia	det. Ib	det. Ib	det. Ia	det. Ia	det. Ib	det. Ib	det. Ib
Seed lectins[c]		PHA + α-AI	PHA + α-AI	PHA + α-AI	PHA + α-AI	*lf*	*lf*	*lf*	*lf*	*lf*
Seed phytate content		*wt*	*wt*	*wt*	*wt*	*wt*	*wt*	*lpa*	*lpa*	*lpa*
Seed weight/mg		550	550	210	243	155	230	—	—	—
Seed coat		Red mottled on beige background	White	Brown	Black	Brown	White	White + black	White + brown + black	White + brown + black

Sequential origin of *lpa* lines = (BAT881 + A55 + G6388) → (938 + 586/8 + *lpa*-280-10 mutant) → *lf* + *lpa* lines (see Section 2 for details).
[a] Submitted to biochemical analyses.
[b] F_3 segregant lines.
[c] Related to lectins present in major amount only (Arcelin, PHA and α-AI).
[d] Plant growth habit according to Singh (1982); det. = determinate.
PHA = phytohaemagglutinin; α-AI = alpha-Amylase Inhibitor; *lf* = lectin-free; *lpa* = low phytic acid; *wt* = wild type.

(without any experimental design) the behavior of first *lf* + *lpa* F_2 plants issued from breeding activity and, again, no major defects were observed. In the present paper, we verify the absence of major agronomic defects in our *lpa* bean materials by the field trial results of three F_3 *lf* + *lpa* bean lines (descendants of the above *lf* + *lpa* F_2 plants), examined for the response to the parameters most negatively associated with the *lpa* trait: seed emergence, average seed weight and grain yield capacity. Preliminary data on high yielding single F_4 white-seeded *lf* + *lpa* plants (last descendants of the above bean materials) are also presented.

2. Materials and methods

2.1. Plant material

Phenotypic and main biochemical traits of the bean cultivars (cvs) and breeding lines tested in this work, are summarized in Table 1. Photoperiod requirement for all is neutral. Agronomic evaluation and seed biochemical characterization of the three F_3 "*lectin-free* (*lf*) + *low phytic acid*" (*lpa*) lines "586/8X61", "586/8X87" and "586/8X147", previously developed at CRA-ORL Research Unit of Montanaso Lombardo (Italy), was the main target of this research. The term "*lectin-free*" indicates the absence in the seed of three major components of the lectin locus: arcelin, phytohaemagglutinin and α-amylase inhibitor (Campion et al., 2009a). The above three *lf* + *lpa* lines originate from the cross [♀586/8 × *lpa*-280-10♂] where "*lpa*-280-10" is a bean mutant carrying both *lf* and *lpa* traits (*lf* + *lpa*) (Campion et al., 2009b) and, "586/8", is an *lf* F_7 white-seeded line (white seed coat = *wsc*), obtained from the cross [♀938 × 865/1♂] (Campion et al., 2009a). The genetic background of the line "938", F_8 *lf*, is [♀BAT881 × (♀A55 × G6388♂)] (Campion et al., 2009a). "BAT881" and "A55" are CIAT accessions kindly provided by Prof. Shree Singh (CIAT, Cali, Colombia). "G6388" is a wild CIAT accession found to carry the *lf* trait (Sparvoli et al., 1994). The average weight of G6388 seeds (obtained in greenhouse) was 70 mg (Campion et al., 2009a) whereas that of *lpa*-280-10 seeds (from field trials) was 159 ± 4.9 mg in 2007 and 168 ± 6.2 in 2008 (Campion et al., 2009b). The cv "Teggia" (borlotto-like) is commercialized by Asgrow (Seminis Division srl – Vegetable Seeds, Italia), and "Sorano" (cannellino-like) is commercialized by Olter Sementi (Blumen srl – Milano, Italia).

2.2. Evaluation of agronomic performance

The nine bean genetic materials reported in Table 1 were evaluated in 2008 in two irrigated field trials, one carried out at the CRA-Research Center of Battipaglia (Salerno, South Italy), and the other at the CRA-Research Unit of Montanaso Lombardo (Lodi, North Italy). BAT881, A55, 938 and 586/8 were used as the controls, being the parents of the new *lf* + *lpa* lines. The commercial cvs "Teggia" and "Sorano" were chosen as the controls cultivated and well adapted to the Italian environments. For each location, two hundred and forty seeds of each cv/line were sown according to a randomized complete block design with four replications (4 replications × 2 locations × 9 cvs/lines). In each experimental plot, formed by two rows placed each at 3.00 × 0.60 m, 60 seeds were sown (30 + 30). For each plot, the following parameters were examined: percent of emerged seedlings surveyed 26 days after sowing, dry seed yield (10–12% water content) expressed as tha^{-1}, plant growth-period duration (no. of days from sowing date to harvest), average seed weight determined on a pool of 200 seeds (Table 2). In 2010, a population of around 700 F_4 plants originating from the seeds of the F_3 *lf* + *lpa* lines tested in 2008, was grown well spaced (30 × 100 cm) without any experimental design in the field of Montanaso L. for generation advancing and selection. In this field we did not cultivate any *wt* control in order to avoid undesirable pollen contaminations. Single F_4 *lf* + *lpa* plants were visually selected for best phenotype, harvested and evaluated for the two most important agronomic quantitative traits, dry grain yield and average seed weight.

Soil composition of Battipaglia was: pH (H_2O) (7.80); sand (268), silt (408), clay (324 g kg^{-1}); active and total limestone (23.5 g kg^{-1}); elemental P (55 mg kg^{-1} – P_2O_5 according to Olsen, 1954); elemental K (0.72 mequiv. 100 g^{-1}); elemental Ca (16.57 mequiv. 100 g^{-1}); elemental Mg (3.31 mequiv. 100 g^{-1}); organic matter (15.5 g kg^{-1}); C/N ratio (6.9); total Fe (17.57 mg g^{-1}); total Zn (26.03 mg kg^{-1}); total P (642 mg kg^{-1}).

Soil composition of Montanaso L. was: pH (H_2O) (5.18); sand (491); silt (369); clay (140 g kg^{-1}); active and total limestone (0 g kg^{-1}); elemental P (59 mg kg^{-1} – P_2O_5 according to Bray and Kurtz, 1945); elemental Ca (4.24 mequiv. 100 g^{-1} corresponding to 849.70 mg kg^{-1}); elemental Mg (0.67 mequiv. 100 g^{-1} corresponding to 81.47 mg kg^{-1}); total Fe (11.18 mg g^{-1}); total Zn (44.80 mg kg^{-1}); total P (485 mg kg^{-1}).

2.3. Biochemical analyses of seed content

The absence of lectins was checked (Campion et al., 2009a) in seed samples expected to have either the *lf* or the *lf* + *lpa* genetic background, all produced in the two field trials. A small seed sample of all the *lf* + *lpa* lines was also checked for high free inorganic phosphorus (Pi) content (fast screening indicating low phytate content – see later) to be sure that no pollen contaminations had occurred during line development. Except Teggia, Sorano and the new *lf* + *lpa* line "586/8X61", all other materials were submitted to the following quantitative analyses of seed composition: dry matter (DM), fiber components (lignin, cellulose and hemicellulose), ash, total phenolics, condensed tannins, trypsin inhibitors, total sapogenins, crude proteins, total iron, total zinc, free phosphorus (free Pi), phytic acid phosphate (PAP). Seed samples of these beans were also submitted to the evaluation of bioavailable iron by applying the *in vitro* Caco-2 cell system. BAT881 and A55 are wild type cultivars (*wt* cvs) for the content of all nutrient/antinutrient compounds examined (*wt* controls). *Lf* lines were also considered a second group of controls, but only for phytate content. Since the *lf* + *lpa* "586/8X87" and "586/8X147" were F_3 recombinant lines, their plot plant population produced a mix of F_4 seeds of three different coat colors (white, black and brown), result of segregation events. Before biochemical analysis, we separated and grouped the seeds for each coat color and considered them as sub-lines (Tables 3 and 4). For each cv/line/sub-line of each location, two seed samples, each one from one field replication, were milled and related flour used for all analyses [(2 *wt* cvs + 2 *lf* lines + 6 *lf* + *lpa* sub-lines) × 2 locations × 2 field replications = 40 flour samples]. For each of these cv/line/sub-line samples, the average seed weight was also determined on a pool of 200 seeds (Table 3). Among the agronomic parameters considered, this was the only one that could be surveyed for each sub-line. For this reason, it was also presented in the frame of biochemical parameters (see data of Table 3 and correlation results of Table 6). Seed weight data of Table 2 were obtained from four field replication data, while those of Table 3 came from two replications.

2.3.1. Dry matter (DM), fiber components (lignin, cellulose and hemicellulose), ash, crude proteins, total phenolics and condensed tannins

These were quantified on samples dried at 60 °C to constant weight and ground in a Cyclotec Model 1093 sampling mill (Foss Tecator AB, Ḧogaña̋s, Sweden) through a 1 mm sieve. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to the method of Goering

Table 2
Factor effect, and mean and standard deviation (SD) of four agronomic parameters surveyed in four cultivars (controls) and two *lf* and three *lf + lpa* lines of bean, all tested in two field trial locations, Battipaglia (Salerno, South Italy) and Montanaso Lombardo (Lodi, North Italy). Mean and SD values presented in tables were calculated as described in Section 2.

	Emerged seedlings (% ± SD)	Dry seed yield (t ha^{-1} ± SD)	Average seed weight[a,b] (mg ± SD)	Plant growth duration[c,d,e] (days ± SD)
Factor effect				
Location	$P = 0.0000$ M > B	ns	$P = 0.0000$ B > M	–
Cv/line	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	–
Cv/line × location	$P = 0.0000$	$P = 0.0001$	$P = 0.0011$	–
Cv/line				
Teggia (control – borlotto)	67.3 ± 9.2 d C	3.28 ± 0.71 cd BCD	566 ± 22.4 a A	87 ± 4.0
Sorano (control – white)	73.7 ± 5.9 cd BC	3.98 ± 0.61 bc B	505 ± 18.2 a A	85 ± 0.0
BAT881 (*wt* parent – brown)	72.3 ± 8.2 cd BC	5.19 ± 0.53 a A	216 ± 6.3 b B	85 ± 0.0
A55 (*wt* parent – black)	85.0 ± 6.0 ab AB	3.89 ± 0.35 bc B	241 ± 8.4 b B	92 ± 4.0
938 (*lf* parent – brown)	76.5 ± 4.9 bcd BC	4.09 ± 0.29 b B	161 ± 9.8 d E	75 ± 0.0
586/8 (*lf* parent – white)	74.6 ± 5.5 bcd BC	2.89 ± 0.36 d CD	189 ± 12.9 c C	91 ± 0.0
586/8X61 (*lf + lpa*)	76.9 ± 7.1 bcd BC	2.79 ± 0.50 d CD	137 ± 8.7 e F	79 ± 0.5
586/8X87 (*lf + lpa*)	82.5 ± 4.6 abc AB	2.64 ± 0.41 d D	167 ± 3.5 d DE	78 ± 0.0
586/8X147 (*lf + lpa*)	91.7 ± 1.9 a A	3.71 ± 0.50 bc BC	179 ± 3.4 c CD	85 ± 0.0

wt = wild type; *lf* = lectin-free; *lpa* = low phytic acid.
Factor effect – *P* significance levels: ($P \leq 0.01$) = highly significant; ($0.01 < P \leq 0.05$) = significant; ($P > 0.05$) = not significant (ns). (M > B) or (B > M) = location mean value of Montanaso L. higher than that of Battipaglia or *vice versa*, respectively.
Cv/line values not sharing a common letter are significantly different at $P \leq 0.05$ (small letters) and $P \leq 0.01$ (capital letters), respectively. Single plant dry grain yield of the most productive cv BAT881 achieved on average 46 ± 29 g at Montanaso L. and 41 ± 20 g at Battipaglia.
[a] ANOVA and Duncan's test analysis applied to transformed [$(10{,}000/X)^2$] data (X = raw data).
[b] Values determined between all (four) field replications
[c] No. of days from sowing date to harvest
[d] Parameter not gathered at Battipaglia
[e] Data not normally distributed.

and van Soest (1976); crude proteins were determined according to Kirsten (1983). Cellulose was estimated by subtracting ADL from ADF and hemicellulose by subtracting ADF from NDF values (Garcia et al., 1997; Claessens et al., 2004). No correction for ash was made. The content of total phenolics in plant extracts was determined by using Folin-Ciocalteu reagent (Singleton and Rossi, 1965) according to the method reported in Taga et al. (1984). All results were expressed as mg gallic acid equivalents/g dry material. Each datum, submitted to statistical analysis, was the mean of three determinations. Total proanthocyanidins (condensed tannins) were determined by the butanol/HCl method, as reported by Porter et al. (1986). All results were expressed as mg delphinidin equivalents/g dry material. Each datum, submitted to statistical analysis, was the mean of three absorbance values.

2.3.2. Total sapogenins

Sapogenins were quantified by an internal standard method as reported in Tava et al. (1993) and Tava and Pecetti (1998). Soyasapogenol B was identified on the bases of soyasapogenols C, D and F obtained as artifact compounds under acidic condition of hydrolyses, by GC/MS and by means of pure reference compounds, and quantified by GC, as previously described (Tava et al., 1993; Tava and Pecetti, 1998). The standard calibration graph was obtained using pure soyasaponin I as representative of sapogenol B saponins and analyzed by the GC/FID using betulinol as internal standard. Results are reported in Table 4 as mean of three independent determinations ± SD.

2.3.3. Trypsin inhibitor activity

This was measured by following the AACC Method 22-40.01 (1999). 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 OD_{410} nm was measured. Inhibitory activity is expressed as μg of trypsin inhibited per mg of flour.

2.3.4. Total iron (Fe) and total zinc (Zn) content evaluation

Five grams flour were dry-ashed and mineralized with 5 ml of HNO_3 65% and 1 ml of H_2O_2 120/130 volumes in a microwave oven as follows: (1) two 5-min steps at 400 W, raising the temperature to 70 °C; (2) two 5-min steps at 560 W, temperature to 125 °C; (3) two 5-min steps at 800 W, 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 a optical ICP (Inductive Coupled Plasma) Spectrometer Perkin Elmer Optima 2100 DV.

2.3.5. Free inorganic phosphate (free Pi)

This was quantified by following the Chen method (Chen et al., 1956): 50 mg of bean seeds flours were extracted with 1 ml of 12.5% TCA, 25 mM $MgCl_2$ solution for 20 min at room temperature and then left stirring overnight at 4 °C. After centrifugation, 100 μl of the supernatant were added to 900 μl of a freshly prepared Chen's reagent [6 N H_2SO_4:2.5% ammonium molybdate:10% ascorbic acid:H_2O (1:1:1:2, v/v/v/v)] and incubated for one h at 50 °C before reading absorbance at 650 nm. A standard curve was performed using a Na_2HPO_4 solution.

2.3.6. Phytic acid phosphate (PAP)

This fraction was determined in a similar way after samples were subjected to a ferric precipitation method as described by Pilu et al. (2003) and expressed as mg/g.

2.3.7. In vitro iron bioavailability assessment

An *in vitro* digestion/Caco-2 cell culture model (Glahn et al., 1998) was used to assess iron bioavailability. With this method, samples are subjected to simulated gastric and intestinal digestion. The intestinal digestion is carried out in cylindrical inserts closed on the bottom by a semipermeable membrane and placed in wells containing Caco-2 cell monolayers bathed in culture medium. The upper chamber was formed by fitting the bottom of Transwell insert ring (Corning) with a 15,000 Da molecular weight cut off (MWCO) membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The

Table 3
Factor effect, and mean and standard deviation (SD) of seven parameters (six biochemical) surveyed in ten bean cultivars/lines/sub-lines (see Section 2 – *biochemical analyses*) tested in two field trial locations, Battipaglia and Montanaso Lombardo. Mean and SD values presented in tables were calculated as described in Section 2. All biochemical values are related to dry matter.

	Hemicellulose (mg g^{-1} ± SD)	Cellulose (mg g^{-1} ± SD)	Lignin (ADL)[a] (mg g^{-1} ± SD)	Ash[b] (mg g^{-1} ± SD)	Total phenolics[c] (mg of gallic acid equivalents g^{-1} ± SD)	Condensed tannins[d] (mg of delphinidin equivalents g^{-1} ± SD)	Average seed weight[e] (mg ± SD)
Factor effect							
Location	ns	ns	ns	$P = 0.0000$ B > M	ns	$P = 0.0013$ M > B	$P = 0.0000$ B > M
Cv/line/sub-line	ns	$P = 0.0001$	ns ($P = 0.0636$)	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$
Cv/line/sub-line × location	ns	ns	ns	$P = 0.0003$	$P = 0.0002$	$P = 0.0149$	$P = 0.0279$
Cv/line/sub-line							
BAT881 (*wt* parent – brown)	18.42 ± 1.88 b	7.63 ± 0.24 c C	1.51 ± 0.34 abc	5.13 ± 0.10 b B	3.09 ± 0.29 cd BC	8.20 ± 0.213 b AB	218 ± 4.2 b B
A55 (*wt* parent – black)	19.81 ± 1.63 ab	8.79 ± 0.71 b BC	1.06 ± 0.29 bc	5.39 ± 0.05 a A	2.92 ± 0.33 cd BC	10.38 ± 0.853 a A	244 ± 8.8 a A
938 (*lf* parent – brown)	21.86 ± 1.75 a	9.27 ± 0.24 b B	1.36 ± 0.36 abc	5.43 ± 0.09 a A	4.02 ± 0.16 a A	6.66 ± 2.000 b BC	162 ±11.3 gh FGH
586/8 (*lf* parent – white)	21.56 ± 1.86 a	9.33 ± 0.52 b B	0.92 ± 0.63 c	5.12 ± 0.02 b B	0.64 ± 0.10 e D	0.18 ± 0.051 d D	196 ± 7.8 c C
586/8X87-white[f] (*lf + lpa*)	21.27 ± 1.96 ab	10.77 ± 0.54 a A	0.88 ± 0.41 bc	5.14 ± 0.05 b B	0.75 ± 0.12 e D	0.21 ± 0.055 d D	148 ± 6.9 i H
586/8X87-black[f] (*lf + lpa*)	20.65 ± 1.20 ab	8.78 ± 0.70 b BC	1.55 ± 0.33 abc	4.87 ± 0.07 cd DE	3.37 ± 0.17 bc AB	3.88 ± 0.369 cd CD	175 ± 5.8 ef DEF
586/8X87-brown[f] (*lf + lpa*)	21.16 ± 1.56 ab	9.02 ± 0.95 b BC	1.78 ± 0.53 ab	4.91 ± 0.17 cd CDE	2.60 ± 0.04 d C	3.17 ± 1.377 cd CD	168 ± 5.7 fg EFG
586/8X147-white[f] (*lf + lpa*)	19.56 ± 1.19 ab	10.88 ± 0.48 a A	1.13 ± 0.60 bc	5.09 ± 0.07 b BC	0.91 ± 0.12 e D	0.23 ± 0.024 d D	155 ± 6.0 hi GH
586/8X147-black[f] (*lf + lpa*)	21.27 ± 0.64 ab	9.34 ± 0.45 b B	1.32 ± 0.35 abc	4.81 ± 0.07 d E	3.82 ± 0.41 ab A	7.72 ± 0.876 b AB	182 ± 2.7 de CDE
586/8X147-brown[f] (*lf + lpa*)	21.04 ± 2.02 ab	8.34 ± 1.14 bc BC	2.22 ± 0.66 a	4.99 ± 0.07 bc BCD	3.31 ± 0.61 c ABC	6.02 ± 1.742 bc BC	189 ± 8.1 cd CD

Factor effect – *P* significance levels: ($P \leq 0.01$) = highly significant; ($0.01 < P \leq 0.05$) = significant; ($P > 0.05$) = not significant (ns); (M > B) or (B > M) = location mean value of Montanaso L. higher than that of Battipaglia or *vice versa*, respectively.
Cv/line/sub-line values not sharing a common letter are significantly different at $P \leq 0.05$ (small letters) and $P \leq 0.01$ (capital letters), respectively.
[a] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $[(X \times 1000)^{0.4}]$
[b] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $(10{,}000/X)$.
[c] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $(X^{1.68})$.
[d] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $(X^{1.4})$.
[e] Values determined between the two field replications submitted to biochemical analyses.
[f] Sub-lines were separated on the basis of the seed coat color; *wt* = wild type; *lf* = lectin-free; *lpa* = low phytic acid; ADL = acid detergent lignin.

Table 4
Factor effect, and mean and standard deviation (SD) of six biochemical parameters surveyed in ten bean cultivars/lines/sub-lines (see Section 2 – *biochemical analyses*) tested in two field trial locations, Battipaglia and Montanaso Lombardo. Mean and SD values presented in tables were calculated as described in Section 2. All values are related to dry matter.

	Trypsin inhibitors[a] ($\mu g\,mg^{-1} \pm SD$)	Total sapogenins[b] ($\mu g\,g^{-1} \pm SD$)	Crude proteins (% ± SD)	Total iron[c] ($\mu g\,g^{-1} \pm SD$)	Total zinc[d] ($\mu g\,g^{-1} \pm SD$)	Ferritin[e] (in Caco-2 cells) ($ng\,mg^{-1}$ cell protein ± SD)
Factor effect						
Location	$P = 0.0000$ M > B	ns	$P = 0.0006$ M > B	$P = 0.0014$ M > B	$P = 0.0002$ M > B	ns
Cv/line/sub-line	$P = 0.0158$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$
Cv/line/sub-line × location	$P = 0.0077$	ns	ns	$P = 0.0214$	ns	ns
Cv/line/sub-line						
BAT881 (*wt* parent – brown)	16.55 ± 0.84 ab	1249 ± 89 ab AB	25.42 ± 0.76 c E	66.1 ± 1.07 cd BC	25.9 ± 0.45 cde BCD	2.68 ± 0.55 b BC
A55 (*wt* parent – black)	15.12 ± 1.91 b	951 ± 35 cd C	25.91 ± 1.54 c CDE	75.6 ± 5.59 ab AB	28.0 ± 1.18 b B	2.40 ± 0.46 bc BC
938 (*lf* parent – brown)	19.09 ± 1.74 ab	1392 ± 69 a A	25.60 ± 0.82 c DE	81.2 ± 10.65 a A	23.6 ± 1.23 f E	2.71 ± 0.26 b B
586/8 (*lf* parent – white)	16.47 ± 0.83 ab	868 ± 92 d CD	29.67 ± 0.62 b B	62.6 ± 1.49 d C	27.4 ± 0.55 bc BC	12.84 ± 0.71 a A [f]
586/8X87-white[g] (*lf + lpa*)	17.29 ± 1.19 ab	708 ± 96 e D	33.73 ± 0.78 a A	79.3 ± 0.31 a A	34.6 ± 1.86 a A	27.97 ± 7.01 a A [f]
586/8X87-black[g] (*lf + lpa*)	18.28 ± 2.62 ab	1019 ± 88 cd BC	29.29 ± 0.69 b BC	69.1 ± 1.60 bc BC	27.4 ± 0.72 bc BC	2.01 ± 0.25 c BC
586/8X87-brown[g] (*lf + lpa*)	18.58 ± 1.78 ab	1061 ± 86 bc BC	27.87 ± 1.52 bc BCDE	66.5 ± 2.33 cd BC	26.8 ± 0.43 bcd BCD	2.31 ± 0.14 bc BC
586/8X147-white[g] (*lf + lpa*)	20.12 ± 0.91 a	847 ± 95 d CD	32.90 ± 0.53 a A	82.2 ± 4.09 a A	32.5 ± 1.67 a A	25.36 ± 4.33 a A [f]
586/8X147-black[g] (*lf + lpa*)	17.62 ± 0.81 ab	875 ± 43 d CD	27.51 ± 1.26 bc BCDE	62.4 ± 2.17 d C	24.7 ± 1.16 ef DE	2.06 ± 0.45 c C
586/8X147-brown[g] (*lf + lpa*)	16.44 ± 2.25 ab	850 ± 19 d CD	28.85 ± 0.48 b BCD	64.5 ± 1.57 cd C	25.2 ± 0.74 cde CDE	2.36 ± 0.50 bc BC

Factor effect – *P* significance levels: ($P \leq 0.01$) = highly significant; ($0.01 < P \leq 0.05$) = significant; ($P > 0.05$) = not significant (ns); (M > B) and (B > M) = location mean value of Montanaso L. higher than that of Battipaglia and vice versa, respectively.
Cv/line/sub-line values not sharing a common letter are significantly different at $P \leq 0.05$ (small letters) and $P \leq 0.01$ (capital letters), respectively.
[a] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $(X^{1.9})$.
[b] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $[(X \times 1000)^{0.01}] \times 100$.
[c] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $(1000/X)$. Total Fe was determined with optical ICP (Inductive Coupled Plasma) Spectrometer Perkin Elmer Optima 2100 DV.
[d] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $[(1000/X)^{1.8}]$. Total Zn was determined with optical ICP (Inductive Coupled Plasma) Spectrometer Perkin Elmer Optima 2100 DV.
[e] ANOVA and Duncan's test analyses applied to transformed data (X = raw data): $[(100/X)^2]$.
[f] The presence of statistical differences at $P \leq 0.05$ and $P \leq 0.01$ were put in evidence by applying a more detailed ANOVA analysis (see Results and Discussion sections and Table 5).
[g] Sub-lines were separated on the basis of the seed coat color; *wt* = wild type *lf* = lectin-free; *lpa* = low phytic acid.

dialysis membrane was held in place using a silicone ring (Web Seal, Rochester, NY). Iron uptake by the cell monolayers is assessed by measuring ferritin concentrations in the cells, as ferritin forms in a consistent response to cell Fe uptake. Six replicates of each Fe bioavailability measurement were performed. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 29. Cells were seeded at densities of 50,000 cells/cm^2 in collagen-treated 6 well plates (Costar Corp., Cambridge, MA). The integrity of the monolayer was verified by optical microscopy. The cells were cultured at 37 °C in an incubator with 5% CO_2 and 95% air atmosphere at constant humidity, and the medium was changed every 48 h. The cells were maintained in Dulbecco's modified Eagle medium plus 1% antibiotic/antimycotic solution, 25 mmol/l HEPES, and 10% fetal bovine serum. Forty eight hours prior to the experiment, the growth medium was removed from culture wells, the cell layer was washed, and the growth medium was replaced with minimum essential media (MEM) at pH 7.0. The MEM was supplemented with 10 mmol/l PIPES, 1% antibiotic/antimycotic solution, 4 mg/l hydrocortisone, 5 mg/l insulin, 5 μg/l selenium, 34 μg/l triiodothyronine, and 20 μg/l epidermal growth factor. This enriched MEM contained less than 80 μg Fe/l. All ingredients and supplements for cell culture media were obtained from Gibco (Rockville, MD). The cells were used in the Fe uptake experiment at 13 days post seeding. On the day of the experiment, 1.5 ml of the digested sample was added to the insert's upper chamber and incubated for 2 h. Then, inserts were removed and 1 ml of MEM was added. Cell cultures were incubated for 22 h at 37 °C. Harvesting of Caco-2 cells and ferritin analysis were as reported in Glahn et al. (1998).

2.4. Statistical analysis of the data

The normality of data distribution was checked for each one of the nineteen parameters examined (fourteen biochemical and five agronomic), by applying the skewness and kurtosis test. Transformed data and those already normally distributed, were submitted to "ANOVA – Two factor complete randomized block design model (MSTATC software)" analysis, in order to evaluate, for each parameter, the effect (*P* value) produced by four factors: replication (effect not shown in tables), location, genetic background of cultivars/lines/sub-lines, and interaction "location × cv/line/sub-line" (see "Factor effect – *P* values" in Tables 2–4). The results of statistical analysis presented in Table 5 were obtained by applying the "ANOVA – One factor complete randomized block design model (MSTATC software)" analysis. In this case, the location effect was

Table 5
Results from ANOVA and Duncan's test analysis obtained by comparing eight genetic materials (four bean lines and four internal controls) which produced the highest amounts of ferritin (in Caco-2 cell system) among all those tested in the work.

Plant material	Type	Ferritin (in Caco-2 cells) ($ng\,mg^{-1}$ cell protein ± SD)
586/8X160-white[a]	*lf + lpa bean*	31.21 ± 2.50 a A
White bean	Internal control	28.35 ± 2.38 ab A
586/8X87-white[b]	*lf + lpa*	27.97 ± 8.87 ab A
586/8X147-white[b]	*lf + lpa*	25.36 ± 6.48 b A
Lentil	Internal control	13.80 ± 2.97 c B
586/8[b]	*lf parent* – white	12.84 ± 3.13 c B
Normal red bean	Internal control	7.81 ± 1.52 d B
High red bean	Internal control	6.85 ± 1.06 d B

Plant material values not sharing a common letter are significantly different at $P \leq 0.05$ (small letters) and $P \leq 0.01$ (capital letters), respectively; SD internal controls: each value was calculated between four replication lab data; SD bean lines/sub-lines: each value was calculated between four agronomic data (the "2 replications × 2 locations" values were considered four replications, including the component produced by the location effect – see Section 2).
[a] *lf + lpa* white seeded bean developed at Montanaso Lombardo together with 586/8X87 and 586/8X147 but not included in the 2008 field trial; in this analysis it was used as one of the internal controls.
[b] Lines/sub-lines tested in the 2008 field trial (see Tables 2–4).

not evaluated, but the "2 locations × 2 replications" values were considered four replications. "Duncan's multiple range test" was applied to rank all means and compare their difference values at significance levels for $P \leq 0.05$ and $P \leq 0.01$ (Tables 2–5).

The groups of data whose distribution could not be normalized were not submitted to ANOVA analysis but their mean and standard deviation (SD) values were all the same reported in tables or in graphics.

Means of each cultivar/line/sub-line presented in tables, were calculated by averaging the "2 locations × no. of replications [4 in field trials (Table 2) and 2 in biochemical analyses (Tables 3–5)]" values.

SD value of each cv/line/sub-line reported in Tables 2–4, was calculated as follows: (1) SD calculation within the field replication values of each location (4 for field trial results in Table 2, and 2 for seed weight and biochemical parameters in Tables 3 and 4); (2) Mean between the two locations' SD values. Mean and SD values of each plant material used in the laboratory as the control for the determination of ferritin (from Caco-2 cells), were calculated from four replication lab values (Table 5). In this test, the SD value of each line/sub-line was calculated by averaging the four "2 replications × 2 locations" values, which, as reported above, were considered four replications (Table 5). Here, it is evident that each SD value included the component produced by the location effect.

The twenty "2 locations × 10 cvs/lines/sub-lines" data of each parameter examined (average values between twenty couples of forty field replication data), were also correlated *vs* those of the other fourteen (average seed weight included) in order to identify possible interrelations (Table 6).

3. Results

3.1. Evaluation of agronomic performance

The *lf* + *lpa* line 586/8X147 showed the highest percentage of plant emergence and a dry seed yield value, not statistically different from that of the second most productive 938. The *lf* + *lpa* line 586/8X87 showed as well a rather high percentage of plant emergence, whereas its dry seed yield, although the lowest, was not statistically different from that of its direct white-seeded parent, *lf* 586/8. The average seed weight values of the two white-seeded *lf* + *lpa* sub-lines (Table 3) were significantly lower than those of related colored *lf* + *lpa* sub-lines, whereas, in comparison to the control *lf* 938, only the white-seeded *lf* + *lpa* 586/8X87 at $P \leq 0.05$ had smaller seeds.

From the *lf* + *lpa* F_4 population cultivated in 2010 at Montanaso Lombardo, thirty-five single plants were visually selected, harvested, and evaluated. Twenty-five of them were white-seeded. The dry seed production of these white-seeded plants ranged between 117 and 267 g whereas their average seed weight (referred to each plant), ranged from 154 to 222 mg. A rough comparison for grain yield could be made with BAT881 single plants of 2008 trials whose average values were 46 ± 29 g at Montanaso L. and 41 ± 20 g at Battipaglia (see also the footnotes of Table 2). The new F_4 plants exhibited Ia or Ib determinate growth habit according to Singh (1982).

3.2. Biochemical analyses of seed content

The absence of lectins, checked in F_4 seed samples having *lf* and *lf* + *lpa* genetic background (harvested from both trials), was confirmed, whereas the free Pi screening performed in the same materials showed the presence, although at a very low rate, of high phytate containing offsprings in the line 586/8X61, indicating that some pollen contamination may have occurred during the steps of

Table 6
Correlation indexes and related significance level found between fifteen parameters (fourteen biochemical) surveyed in ten bean cultivars/lines/sub-lines (see 'Section 2 – *biochemical analyses*") tested in two field trial locations, Battipaglia and Montanaso Lombardo. For each parameter, correlated values were 20 means each one calculated by averaging the two field replication values (see Section 2). All correlated biochemical values are related to dry matter.

	Trypsin inhibitors	Ferritin	Total Zn[a]	Total Fe[a]	Free Pi	Crude proteins	Total sapogenins	PAP	Condensed tannins	Total phenolics	Cellulose	Hemi-cellulose	Lignin (ADL)	Ash
Average seed weight	−0.629**	−0.508*	−0.413 $P=0.070$	−0.421 $P=0.064$	−0.560**	−0.632**	ns	+0.665**	+0.510*	ns	−0.583**	ns	ns	ns
Ash	ns	ns	ns	ns	−0.532*	ns	ns	+0.691**	ns	ns	ns	ns	ns	
Lignin (ADL)	ns	−0.458*	−0.482*	ns	ns	ns	ns	ns	ns	+0.420 $P=0.065$	−0.585**	ns		
Hemicellulose	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
Cellulose	ns	+0.730**	+0.681**	+0.574**	+0.398 $P=0.082$	+0.659**	ns	ns	−0.595**	−0.605**				
Total phenolics	ns	−0.798**	−0.731**	ns	ns	−0.686**	+0.516*	ns	+0.721**					
Condensed tannins	ns	−0.708**	−0.538*	ns	ns	−0.722**	ns	ns						
PAP	ns	ns[b]	ns	ns	−0.901**	−0.572**	+0.509*							
Total sapogenins	ns	−0.556*	−0.583**	ns	−0.522*	−0.737**								
Crude proteins	ns	+0.849**	+0.814**	ns	+0.578**									
Free Pi	ns	ns	ns	ns										
Total Fe[a]	+0.458*	+0.518*	+0.573**											
Total Zn[a]	ns	+0.868**												
Ferritin	ns													

ns = not significant. The *P* values of the "average seed weight/Total Zn", "average seed weight/total Fe", "Cellulose/free Pi", and "lignin/total phenolics" correlations, were near to be significant.
[a] Total Fe and Zn: determined with optical ICP (Inductive Coupled Plasma) Spectrometer Perkin Elmer Optima 2100 DV; ADL = acid detergent lignin; PAP = phytic acid phosphorus.
[b] The absence of *lpa* colored beans' values in the data population produces a negative highly significant correlation index (−0.787**).
* Significant at $0.01 < P \leq 0.05$.
** Significant at $P \leq 0.01$.

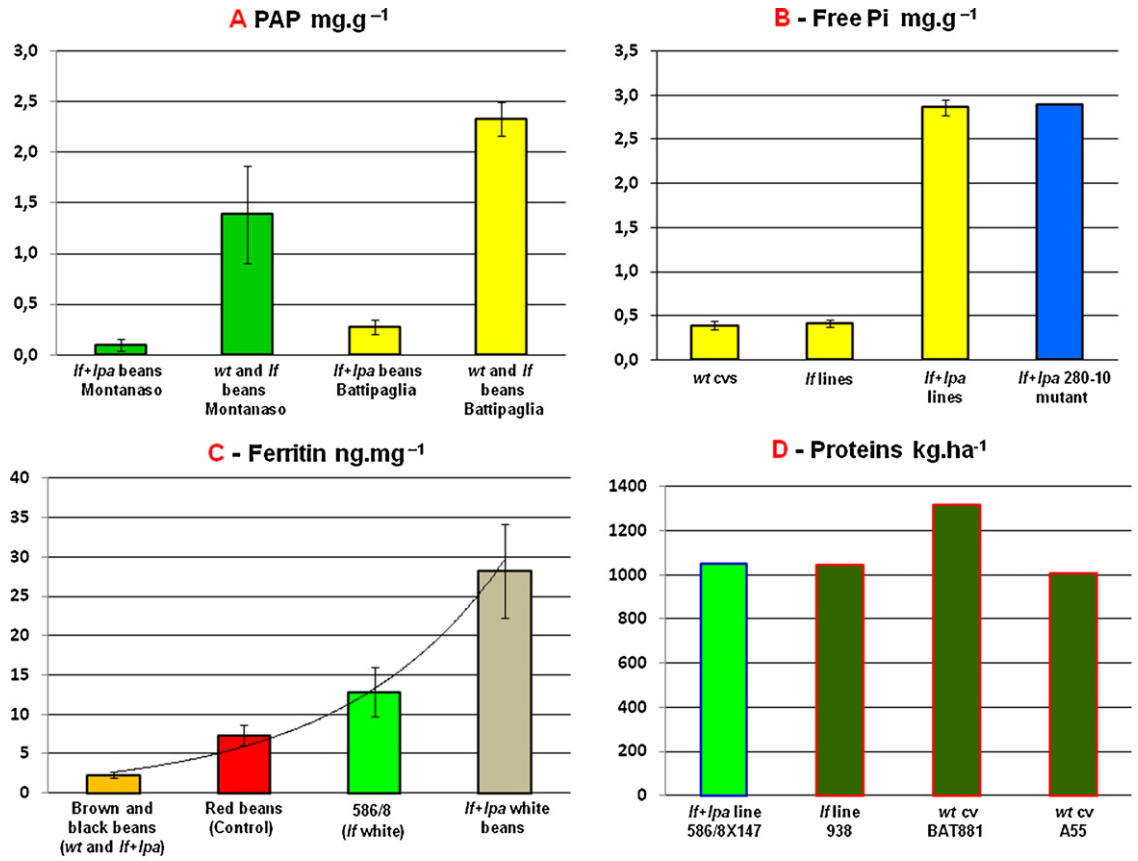

Fig. 1. PAP = phytic acid phosphorus (A), Free Pi = free inorganic phosphorus (B), ferritin formation in Caco-2 cells quantified as ng mg^{-1} cell protein ± SD (C), related to seed flour of different genetic backgrounds of common bean as follows: *wt* cvs = *wild types* cultivars BAT881 and A55; *lf* = *lectin-free* materials related to the brown and white seeded lines 938 and/or 586/8, respectively; *lf* + *lpa* = "*lectin-free* + *low phytic acid*" materials related to white, and/or black and/or brown seeds of the two lines 586/8X87 and 586/8X147. Histogram values of A–B–C were obtained by grouping data on the basis of their homogeneity defined by beans characteristics and/or statistical analysis (Duncan's test ranked values). All histogram values are related to dry matter. Standard deviation values shown by strokes visible in A–B–C, were calculated by averaging the SD mean values of the two locations. Red beans showed in the red histogram of C were used as one of the internal controls. Protein yield (D) of the *lf* + *lpa* line 586/8X147 was calculated as "total production of each seed color class × related protein%" pondered values, all related to dry matter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lpa line development. For this reason, this line was not submitted to biochemical analyses. PAP and free Pi data were not normally distributed (as expected) and could not be corrected by transformation. Thus, they were synthetically reported graphically without any statistical analysis (PAP in Fig. 1A and free Pi in Fig. 1B). PAP seed content of the *lf* + *lpa* lines (average values between all *lf* + *lpa* sub-lines data for both locations) was approximately ten times lower (89% less – actually this reduction can vary from 85 to 91% depending on the genotype and location) than that of the four controls (average values between *lf* lines 938 and 586/8, and *wt* cvs BAT881 and A55 for both locations), whereas free Pi seed content was approximately seven times higher. The free Pi level of *lf* + *lpa* lines was equal to that of *lpa* 280-10 mutant (*lpa* control – Fig. 1B) produced in 2007 at Montanaso L. (Campion et al., 2009b).

Probably due to the too high dispersion and low homogeneity of the ferritin data (Table 4), no statistical differences were shown between the *lf* white control 586/8 (high phytate content) and the two *lf* + *lpa* white lines (586/8X87 and 586/8X147). By contrast, a more detailed ANOVA analysis performed on a restricted group of eight samples (five internal controls, the *lf* white 586/8, and the two *lf* + *lpa* white 586/8X87 and 586/8X147), all exhibiting higher and more homogeneous values of ferritin, generated high statistical differences ($P \leq 0.01$ – Table 5). In this analysis, we excluded the bean samples whose ferritin values were less than 5 ng mg^{-1} cell protein (*wt*, *lf* and *lf* + *lpa* colored beans), and included those of different internal controls (normal red and high red beans, lentil, a white bean, and a new *lf* + *lpa* white-seeded bean line developed at Montanaso L. which was not included in the 2008 field trial), whose ferritin values ranged between 6.8 and 31.2 ng mg^{-1} cell protein.

From the correlation results of Table 6, it is possible to identify two different groups of nutritional parameters which, based on their accumulation characteristics, tend to correlate positively within them and negatively toward those of the other group. One group includes all the compounds having a direct nutritional function (crude proteins, total Fe, total Zn and free Pi), the *in vitro* iron bioavailability index, and cellulose. The second group includes all those compounds which exhibit antinutritional activity (total phenolics, condensed tannins, PAP) as well as lignin and saponins, this last indirectly expressed by sapogenin amount.

4. Discussion

4.1. Agronomic performance of *lf* ± *lpa* bean lines

It is well known that in all crops so far examined, the selection of *lpa* mutations correlates with the onset of a number of agronomic defects especially when phytate reduction reaches high percentages (usually, over 70%) (Raboy et al., 2000; Pilu et al., 2005; Bregitzer and Raboy, 2006). It is also of common knowledge that a number of plants arising from crosses of new genetic materials, usually exhibit agronomic defects at F_3 generation, due to the presence of a high heterozygosity (from F_3 generation they usually

need to be selected). All this notwithstanding, no major depressing effects were observed in our *lf*+*lpa* materials and their field performance was comparable or even better than that of related *lf* and *wt* parents. On this matter, we found out that seedling emergence was definitely higher in the *lf*+*lpa* lines (Table 2), whereas grain and protein yield (Table 2 and Fig. 1D, respectively) were comparable to those of 938 and A55. Average seed weight was significantly reduced only in the *lf*+*lpa* 586/8X61 (Table 2), but not in the other two lines. Hence, it is conceivable that seed size reduction in this case cannot be attributed to the *lpa* trait influence, but rather to a particular genetic background of the line 586/8X61. Moreover, we should also consider that all *lf*+*lpa* materials originated from a wild lectin-free CIAT accession (G6388) whose average seed size was extremely low (≈70 mg – see Section 2). Finally, encouraging although indicative data were also obtained from the twenty five F_4 *lf*+*lpa* white-seeded plants selected and harvested in 2010, which appeared to satisfy our nutritional targets. Their good dry seed yield and discrete average seed weight range values (117–267 g and 154–222 mg, respectively), indicate that these two parameters can easily be improved through further selection and/or breeding activity in this type of bean.

The results presented in this work proves the lack of major defects directly visible in *lpa* bean lines cultivated in good agronomic conditions (irrigated soils) but do not exclude the presence of minor defects or of possible major defects arising from particular stressed agronomic conditions [*i.e.* like those reported in barley (Bregitzer and Raboy, 2006)]. The individuation of these second group of defects in our *lpa* materials can be done by examining the agronomic performance of selected advanced lines (F_7–F_{10}, BC_4F, *etc.* generation level) in multiple year-location field trials which, so far, we could not realize. Up to now we can only state that, if present, they do not preclude the positive effect of selection pressure applied during generation advancing (now in progress).

A possible hypothesis that could explain the lack of major agronomic defects in *lpa* 280-10 mutant bean plants may be as follows: the bean *lpa*-280-10 mutation is due to a defective ABC transporter of the MRP family *(PvMRP1*), necessary for phytic acid vacuole compartmentalization (Panzeri et al., 2011). In the same work it is also shown that, in common bean, likewise in soybean but contrary to cereals, a second MRP paralog (*PvMRP2*) exists, and that it is poorly expressed in seeds but not in other plant parts. Thus, the expression of the *PvMRP2* gene might complement the effects of the defective *Pvmrp1* gene in vegetative tissues and further explain the absence of negative phenotypes. A further hypothesis is that a determinant role might be played by the different seed anatomy and location of the phytic acid (and cations bound to it) accumulation: in cotyledons (bean and soybean) instead of in the aleurone layer or embryo (cereals). Doria et al., 2009 showed that phytic acid performs an antioxidant function during maize seed maturation and storage. It prevents iron ions from being free to undergo Fenton reaction generating ROS (Reactive Oxygen Species). Since it is conceivable that oxidative damages to aleurone or embryo cells would preclude germination and growth much more than damages to cotyledon tissues, this function is probably more "crucial" in aleurone and embryo tissues than in cotyledons.

4.2. Nutrient and antinutrient accumulation in *lf* ± *lpa* bean seed

The strong genetic reduction of the two major antinutrients phytic acid and condensed tannins, together with the elimination of the three major lectins in our new *lf*+*lpa* beans, interestingly generated a reduction of total phenolics and lignin as well as of saponins, while it led to an increase of all compounds having an important nutrient function (crude proteins, free Pi, total zinc and, indirectly, also total iron – see correlation results of Table 6). The increase appears rather high: crude proteins and total zinc around + 30%, free Pi + 600% (Table 4 and Fig. 1B). Higher accumulation in the seed was unexpected for protein and zinc, and was expected for Pi, as previously found out in *lpa* mutants of other species. In relation to iron content in *lf*+*lpa* white-seeded beans, we cannot consider it a real increase, but rather a capacity to reach the highest levels, comparable to those of the best control line, the *lf* 938. This is important because it demonstrates that iron may be accumulated at normal amounts in *lpa* seeds, in spite of the very low phytic acid content. Concerning the fiber components, it is interesting that cellulose accumulation is positively correlated with three important nutrients (crude proteins, total iron and zinc), and negatively with two antinutrients (tannins and total phenolics) and with lignin, all in a highly significant way ($P \leq 0.01$). Finally, the accumulation of saponins resembles that of the main antinutrients (Table 6).

From an agronomic point of view, the negative correlation between nutrients and antinutrients found in the present work, is not less important than the other two aspects (agronomic performance and iron bioavailability). In fact, it might reveal a capacity of white-seeded *lf*+*lpa* beans to produce superior amounts of protein per ha as a result of an energy saving or recovery from the reduced synthesis of antinutrients. The exceptional high seed production of the twenty five single F_4 white-seeded *lf*+*lpa* plants is consistent with this hypothesis.

4.3. In vitro iron bioavailability of *lf* ± *lpa* bean line seeds

The amount of ferritin produced by the *in vitro* cultured Caco-2 cells is proportional to that of bioavailable iron present in the medium, thus by feeding cells with bean flour digestate (Glahn et al., 1998) we generated relative comparisons of bioavailable Fe in the *lf*+*lpa* bean seeds (Tables 4 and 5 and Fig. 1C). Our results indicate that the genetic reduction/elimination of the three antinutrients lead to a strong increase of iron bioavailability in the white coated *lf*+*lpa* beans (twelve times higher than that of *wt* colored seeds), while no increase was found in *lf*+*lpa* beans with brown and black seeds, in comparison to the *wt* controls. In this case, it is evident that tannins contained in the *lf*+*lpa* colored seeds (more than 3 mg of delphinidin equiv. g^{-1} – Table 3), undo the benefits of phytate reduction in increasing iron bioavailability. Conversely, these benefits are very high when tannins are nearly absent, as demonstrated by the highly significant difference ($P \leq 0.01$) between the white parent *lf* 586/8 (high phytate content) and its two white *lf*+*lpa* descendants (586/8X87 and 586/8X147) (Table 5 and Fig. 1C). The negative effect of tannins and in general of all antinutrients on ferritin formation is also confirmed by correlation results (Table 6), whose indexes are all negative.

According to a few authors (Glahn et al., 2002; Bohn et al., 2008), the inhibitory effect exerted *in vitro* by tannins is much stronger than that exerted by phytates. A similar though not identical response has been previously observed *in vivo* (trials carried out in humans), where the presence of at least one of the two antinutrients (tannins or phytate) was found to fully inhibit iron bioavailability of tested meals (Petry et al., 2010), while the absence of both antinutrients was effective in increasing it by 3.4 fold.

From our study, it is thus clear that only the white-seeded *lf*+*lpa* beans are endowed with nutritional characteristics able to satisfy the most important nutritional needs.

4.4. General remarks

As far as we know, a 90% reduction of phytic acid together with 98% reduction of condensed tannins and the absence of major lectins in one genetic background, represents a rather extreme physiological condition, never described so far in common bean. The salient results obtained in the present work are mainly three: (1) the agronomic performance of the *lf*+*lpa* bean lines at F_3

generation is comparable to that of their control parents, showing the absence of major defects; (2) the genetic removal/reduction of antinutrients in the seed is counterbalanced by a significant increase of the content of important nutrients; (3) the level of iron bioavailability detected *in vitro* is several times higher on average in the *lf+lpa* white-seeded bean lines (containing low amounts of both polyphenols and phytate) than in all other wild type and *lf+lpa* colored genotypes. The first two findings are very important from the agronomic and nutritional point of view, respectively, while the third is of huge interest in the field of mineral bioavailability, thus of biofortification. To this regard, the *lf+lpa* white bean seeds could meet the human food prerequisite as defined by Petry et al. (2010) according to which higher amounts of iron could be bioavailable only when tannins and phytate are both present at low levels in the bean seed. This becomes even more important since the results obtained recently in humans by Petry et al. (2012) show that phytate and polyphenols normally contained in the biofortified beans undo completely the expected benefit in terms of higher bioavailability due the abundant levels of iron contained in their seeds. Since previous works have shown a good correlation between the results provided by the Caco-2 cells system and those obtained in humans (Yun et al., 2004; Fairweather-Tait et al., 2005; Glahn, 2009), we can easily infer that, in humans, the *lf+lpa* white beans will efficaciously provide higher amounts of bioavailable iron than normal beans. If this will be experimentally confirmed, considerable and important improvements could be further achieved through the realization of a Research Project aimed to the development of a new bean carrying in its genetic background both the positive traits of our lines (*lf+lpa+white seed coat*) and those of the biofortified beans developed by CIAT (very high amounts of iron and zinc in the seed, presence of important disease resistances). This project seems now feasible and might bring the nutritional value of bean nearer to that of meat. This appears to be of considerable importance because meat, in spite of its high nutritional quality, shows significant drawbacks such as the high energy input required to produce it (estimated to be more than five fold than that required to produce bean grain) and the related enormous amount of animal waste to deal with. Indeed, the increased global population requires a much more ecologically sustainable protein source that should be able to replace meat in a balanced diet.

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