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Detection and exploitation of white lupin (*Lupinus albus* L.) genetic variation for seed γ-conglutin content

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Summary

The seed γ-conglutin protein fraction of white lupin has particular pharmacological interest, but its industrial production is hindered by low content in the seed. This study provides an unprecedented assessment of genotypic and environmental variation for seed content and production of γ-conglutin, exploring also the ability of Near-Infrared Spectroscopy (NIRS) to predict seed γ-conglutin content. Significant ($P < 0.01$) genetic variation for seed γ-conglutin content emerged among ten genotypes (cultivars or breeding lines) across three environments (range: 1.59-2.02 %) and five genotypes in other two environments (range: 1.47-1.80 %). Genotype variation was found also for seed protein content and γ-conglutin proportion on total protein, the latter trait having higher impact than the former on genotype variation for seed γ-conglutin content. The production of γ-conglutin per unit area was affected also by genotype yielding ability beside genotype seed γ-conglutin content. No genotype × environment interaction was detected for any γ-conglutin trait. NIRS-based prediction based on cross-validations was only moderately accurate for seed γ-conglutin content ($R^2 = 0.66$), while being accurate for seed protein content ($R^2 = 0.95$). In conclusion, breeding for higher seed γ-conglutin content is feasible using data from very few test sites and, to some extent, NIRS-based predictions.

Introduction

Greater cultivation of rain-fed cool-season grain legumes could improve the sustainability of European agriculture in many respects (NEMECEK et al., 2008). White lupin (*Lupinus albus* L.) is a traditional food legume with good potential as a high-protein feed crop in several European regions (LUCAS et al., 2015), owing to its moderately high grain yield, outstanding seed protein content, and good content of essential amino acids (PAPINEAU and HUYGHE, 2004; SUJAK et al., 2006; ANNICCHIARICO, 2008). In addition the white lupin seed contains 8-12% of oil with excellent nutritional characteristics (BOSCHIN et al., 2008), and possesses various properties that make it particularly useful as an ingredient of functional, nutraceutical or healthy foods (ARNOLDI, 2005; DURANTI et al., 2008). The γ-conglutin protein fraction has particular interest in this respect, owing to its proved ability to control glycaemia via interaction and binding with insulin and its insulin-mimetic properties (BERTOGLIO et al., 2011; LOVATI et al., 2012). A patent, which awaits commercial exploitation, has been granted for the pharmacological use of γ-conglutin for the treatment of type II diabetes (MORAZZONI and DURANTI, 2004).

Large-scale production of white lupin seed γ-conglutin is hindered by its modest content in the seed, which represents only about 4-5% of the total protein (DURANTI et al., 2008). Optimizing its industrial production requires information on the extent of genetic, environmental, and genotype × environment interaction effects that may influence its content. Earlier work on other seed quality traits of white lupin, such as oil, tocopherol or alkaloid contents, revealed that the relative size of these effects may vary largely depending on the specific trait (ANNICCHIARICO et al., 2014), with implications for crop improvement strategies and optimal production environments. Despite its practical importance, we found no published information on the extent of genetic and environmental variation for seed γ-conglutin of white lupin. If exploitable genetic variation existed, it would be important to know whether it is associated mainly with overall protein content or with the proportion of γ-conglutin on total protein. Only the latter case would prompt selection programs to specific analyses of genotype seed γ-conglutin content (which are more expensive than protein content analyses). In that case, Near-Infrared Spectroscopy (NIRS) procedures could be explored as a means to reduce the costs of seed γ-conglutin analyses for plant breeding material or seed lots from different production environments. NIRS has been already used to predict seed coat proportion in intact seeds of lupin (ALOMAR et al., 2010) and contents of amino acids and protein in the seed (BISTON et al., 1983; KAFFKA, 1988).

The main objective of this study was generating information on the extent of genetic and environmental variation for γ-conglutin in white lupin seed. In addition we report preliminary information on the relationship of seed γ-conglutin with total protein content and the ability of NIRS procedures to predict seed γ-conglutin content.

Materials and methods

Plant material and cultivation

Seven elite breeding lines (7-50, 685, 722, B-17, B-20, MB-29 and MB-38) issued by a white lupin breeding program for climatically-contrasting Italian regions that is described in ANNICCHIARICO et al. (2011), and three commercial varieties (Lucky, Rex, Rumbo), were grown in three autumn-sown field experiments with the aim to gather preliminary information on the genetic variation for seed γ-conglutin. The experiments were performed in the subcontinental-climate site of Lodi (Northern Italy) in the seasons 2011-12 and 2012-13, and the Mediterranean-climate site of Sanluri (Sardinia) in 2012-13. Climate and soil characteristics of these test environments are reported in Tab. 1. White lupin underwent low-temperature stress during winter in Lodi and terminal drought stress mainly in Sanluri (featuring modest spring rainfall), in agreement with the general climatic features of the sites. Soil characteristics, including active lime < 0.5% and pH < 7.5, were always suitable for lupin cultivation (PAPINEAU and HUYGHE, 2004). These experiments adopted a randomized complete block design with three replications, plots of 3 m^2 size, and 20 and 30 germinating seeds/m^2 in 2011-12 and 2012-13, respectively. In these and following experiments, the seed was inoculated with NPPL HiStick (Becker Underwood) before sowing. Seed γ-conglutin analyses were carried out for each genotype-environment combination on a seed sample of 50 g that was obtained by bulking same amounts of seed from each experiment replicate.

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Tab. 1: Climate and soil characteristics, and mean white lupin grain yield and seed γ-conglutin content, for two test locations (Lodi, Northern Italy; Sanluri, Sardinia) in different growing seasons.

	Lodi			Sanluri	
Variable	**2011-12**	**2012-13**	**2013-14**	**2012-13**	**2013-14**
Rainfall Nov-Feb (mm)	124	365	427	266	432
Rainfall Mar-Jun (mm)	222	598	270	148	163
Lowest winter temp (°C)	-17.0	-11.3	-5.7	-2.3	0.2
Mean temperature Nov-Feb (°C)	2.4	5.0	4.9	10.8	16.1
Mean temperature Mar-Jun (°C)	15.7	14.2	16.6	11.2	16.6
Soil active lime (%) [a]	~0	~0	~0	0.3	2.6
Soil pH	6.2	6.3	6.2	7.4	8.1
Grain yield (t/ha) [b]	6.41	5.10	3.30	6.24	1.15
Seed γ-conglutin content (% w/w) [b]	1.96	1.64	1.73	1.88	1.52

[a] According to Drouineau (1942).
[b] Averaged across 10 genotypes in 2011-12 and 2012-13, and five genotypes in 2013-14.

Two breeding lines that displayed high and moderate seed γ-conglutin content in these experiments (lines 7-50 and MB-38, respectively), and three varieties (Lucky; Multitalia; Rumbo), were grown in autumn-sown field experiments in Lodi and Sanluri in the season 2013-14. To further enhance the environmental differences between testing sites, the evaluation in Sanluri was performed in a field whose soil active lime and pH (Tab. 1) were definitely higher than those required for optimal lupin growth (Papineau and Huyghe, 2004), unlike Lodi. Grain yield in Lodi was limited by biotic stresses (particularly *Fusarium* spp.). Rainfall and temperature patterns were relatively favourable in both locations (Tab. 1). These experiments adopted a randomized complete block design with four replications, plots of 6 m^2 size, and 30 germinating seeds/m^2. Genotype grain yields were assessed in all field replicates, whereas seed γ-conglutin and protein contents were assessed on two replicates per experiment using 50 g seed samples.

Chemical and NIRS analyses

Lupin seed samples were ground to 1 mm particle-size in three grinding steps using a "Pulverisette 14" Variable Speed Rotor Mill (Fritsch GmbH, Idar-Oberstein, Germany) with different sieve rings.

The γ-conglutin content of each ground sample was assessed by Indena's proprietary HPLC-UV method, whose details cannot be disclosed completely. A single, exhaustive extraction of γ-conglutin from the lupin flour was obtained in a Tris-Glycine buffer (extraction solvent/flour ratio: 200). After centrifugation, the supernatant was analyzed twice on a reverse phase HPLC, using an apolar stationary phase butyl silicagel and an acetonitrile/water/TFA gradient. Detection was carried out by ultraviolet at 210 nm. Quantification was carried out against a γ-conglutin reference standard. This method was assessed for linearity (0.01-0.4 mg/ml), precision (relative standard deviation < 4%), and stability of the sample solution (up to 48 hours). Seed protein content was determined as described by Kjeldahl (1883).

NIRS analyses were performed on 46 seed samples, which included: (i) 30 genotype-environment combinations of experiments in the seasons 2011-12 and 2012-13; (ii) ten genotype-environment combinations of experiments in 2013-14 (using seed of the first field replicate); (iii) six additional seed samples relative to other breeding lines that were evaluated only in Lodi in 2011-12 or 2013-14. Two ground seed replications of each sample were analyzed in Petri dishes (re-blending and re-loading the dish between measurements), averaging results of the two measurements. We used a FTNIR spectrometer (NIRFlex N500, Büchi, Italy) in the whole NIRS range (10,000-4,000 cm^{-1}). NIRS spectra were collected in reflectance mode, adding 64 scans with a 4 cm^{-1} resolution. Spectra were converted in absorbance mode before data analysis.

Statistical analysis

Genotype data of seed γ-conglutin from Lodi and Sanluri in the seasons 2011-12 and 2012-13 underwent an analysis of variance (ANOVA) aimed to compare the genotype means across the three environments and the environment means, using genotype × environment interaction as the error term.

Plot data of seed γ-conglutin and protein contents, proportion of γ-conglutin on total protein, and grain and γ-conglutin dry matter (DM) yield from Lodi and Sanluri collected in the season 2013-14 underwent an ANOVA aimed at assessing genotype, environment and genotype × environment interaction effects and comparing genotype means across environments. Genotype γ-conglutin DM yield on a plot basis was computed as the product of plot yield by genotype seed γ-conglutin content on the site. Data from these environments were also used for simple and multiple regression analyses of seed γ-conglutin content as a function of protein content and/or γ-conglutin proportion of total protein. All ANOVA and regression analyses were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA).

NIRS calibrations to quantify the seed contents of γ-conglutin and protein were calculated using the software PLS Toolbox (Eigenvector Research Inc, Manson, WA, USA). We used different spectra pretreatments to improve NIRS calibration performances. Owing to the limited number of available samples, each calibration curve was assessed through cross-validations that used 11% of the spectra for predictions, repeating the procedure nine times using contiguous data blocks.

Results

Significant ($P < 0.01$) genetic variation among ten genotypes emerged for mean seed γ-conglutin content across three test environments in the cropping seasons 2011-12 and 2012-13. Genotype values ranged from 1.59% to 2.02%, both exhibited by breeding lines. Control varieties displayed moderate to low values (1.60-1.83%).

On average, the environment of Lodi 2012-13 displayed lower seed γ-conglutin content (1.64±0.05%) than Lodi 2011-12 (1.96±0.05%) and Sanluri 2012-13 (1.88±0.05%) ($P < 0.05$). One breeding line (7-50) with high seed γ-conglutin content (1.95%; Tab. 2), and a second line (MB-38) with moderate γ-conglutin content (1.72%), were selected for further testing in Lodi and Sanluri in 2013-14 along with three commercial varieties.

The ANOVA performed on data from the season 2013-14 detected no genotype × environment interaction for every γ-conglutin trait, namely, seed γ-conglutin content, proportion of γ-conglutin on total protein, and γ-conglutin yield per unit area. In contrast, genotype × environment interaction ($P < 0.05$) was detected for seed protein content and grain yield. Large variation for seed γ-conglutin content of genotypes across environments was found also in this data set ($P < 0.01$), with values ranging from 1.47 % in Multitalia to 1.80 % in the line 7-50 (Tab. 2). The ranking of four genotypes for seed γ-conglutin content across two environments in 2013-14 was identical to that across three environments in 2011-12 and 2012-13 (Tab. 2), confirming the high consistency of genotype responses across environments for this trait.

Significant ($P < 0.01$) genotype variation across environments of the season 2013-14 emerged for all other traits. Line 7-50 achieved top-values of seed γ-conglutin content via high values of both seed protein content and proportion of γ-conglutin on total protein (Tab. 2). The variety Rumbo exhibited low seed γ-conglutin content because of low proportion of γ-conglutin on total protein, since its protein content was the highest one (Tab. 2). Results of simple and multiple regression analyses, which are summarized in Tab. 3, confirmed that: (i) seed γ-conglutin content of the genotypes was largely determined by the proportion of γ-conglutin in the protein fraction (with 81% of genotype variation explained by this variable); (ii) additional information on seed protein content had some value for predicting seed γ-conglutin content (as showed by its significance in the multiple regression model).

The production of γ-conglutin per unit area of the genotypes was also influenced by genotype grain yielding ability besides seed γ-conglutin content. The variety Lucky and the line 7-50, which combined high values of both variables, were top-ranking for γ-conglutin yield (Tab. 2).

On average, the unfavorable cropping environment of Sanluri in 2013-14 exhibited, in comparison to Lodi in the same season, not only almost three-fold lower grain yield but also lower seed γ-conglutin content (Tab. 4). The latter response was the result of distinctly lower seed protein content, while the proportion of γ-conglutin on total protein did not differ between environments (Tab. 4).

A NIRS-based calibration curve for seed γ-conglutin content was established, which held five latent variables and used a standard normal variate and a gap segment 2nd derivative as pretreatments to process the spectra. It displayed moderate predicting ability based on cross-validations ($R^2 = 0.66$, with a root mean square error of 0.14). Predictions for individual observations tended to shrink the range of γ-conglutin content relative to observed values (Fig. 1). A NIRS-based calibration curve for seed proteins content, which used four latent variables and a standard normal variate plus Savitzky-Golay 1st derivative algorithm as pretreatments, achieved much higher prediction ($R^2 = 0.95$, with a root mean square error of 0.81).

Tab. 3: Ability of simple and multiple linear regressions models to explain seed γ-conglutin content variation of five white lupin genotypes across two climatically-contrasting Italian environments as a function of seed protein content and/or γ-conglutin proportion on total protein.

Model variables	R^2	P [a]
γ-conglutin proportion on total protein	0.81	0.05
Seed protein content	0.01	0.05
γ-conglutin proportion on total protein + Seed protein content	0.99	0.05 / 0.05

[a] P level significance of explanatory variables in the model.

Discussion

Our study provides an unprecedented assessment of the extent of genotypic and environmental variation for seed content and production of seed γ-conglutin in white lupin. Its indications, while being valuable in the absence of other information, cannot be considered as conclusive, owing to the fairly limited sample of genotypes and test environments they are based upon.

Our findings indicate that white lupin selection for high seed content of γ-conglutin is feasible, on the basis of (i) the large genetic variation detected for this trait, and (ii) the consistency of genotype differences across environments. The latter result is supported by the lack of genotype × environment interaction in the ANOVA for every γ-conglutin trait, and the consistent ranking of four genotypes for seed γ-conglutin content across two sets of environments. The consistency of genotype responses for seed γ-conglutin content facilitates genotype selection, which may rely on results from very few test environments, and ensures that selection gains are maintained in

Tab. 2: Mean values over two climatically-contrasting Italian locations of seed γ-conglutin and protein contents (in % w/w) measured by HPLC and Kjeldahl methods, respectively, and grain and γ-conglutin dry matter (DM) yield, for five white lupin genotypes.

Genotype	γ-conglutin % [a]	Protein % [a]	γ-conglutin % on protein [a]	Grain DM (t/ha) [a]	γ-conglutin DM (kg/ha) [a]	γ-conglutin % [b]
7-50	1.80 a	34.15 b	0.053 a	2.35 b	42.4 ab	1.95 a
Lucky	1.73 ab	32.50 c	0.053 a	2.77 a	47.8 a	1.83 ab
MB-38	1.61 bc	33.77 b	0.048 ab	1.84 c	29.6 c	1.72 ab
Rumbo	1.51 c	35.83 a	0.042 b	1.59 c	24.0 d	1.60 b
Multitalia	1.47 c	32.15 c	0.046 b	2.59 ab	38.1 b	-
SE (DF) [c]	0.04 (8)	0.34 (8)	0.002 (8)	0.10 (24)	1.8 (24)	0.09 (18)

Column means with different letter differ at $P < 0.05$ according to Duncan's test.
[a] Over two environments (Lodi and Sanluri, 2013-14).
[b] Over three environments (Lodi 2011-12 and 2012-13; Sanluri, 2012-13).
[c] SE, standard error of genotype mean; DF, associated degrees of freedom.

Tab. 4: Mean values over five white lupin genotypes of seed γ-conglutin and protein contents (in % w/w) measured by HPLC and Kjeldahl methods, respectively, and grain and γ-conglutin dry matter (DM) yield, for two contrasting cropping environments (Lodi, northern Italy; Sanluri, Sardinia) in the season 2013-14.

Environment	γ-conglutin %	Protein %	γ-conglutin % on protein	Grain DM (t/ha)	γ-conglutin DM (kg/ha)
Lodi	1.73 a	37.69 a	0.046 a	3.30 a	52.2 a
Sanluri	1.52 b	29.68 b	0.051 a	1.15 b	18.9 b
SE (DF) [a]	0.05 (2)	0.36 (2)	0.002 (2)	0.12 (6)	2.4 (6)

Column means with different letter differ at $P < 0.05$.
[a] SE, standard error of genotype mean; DF, associated degrees of freedom.

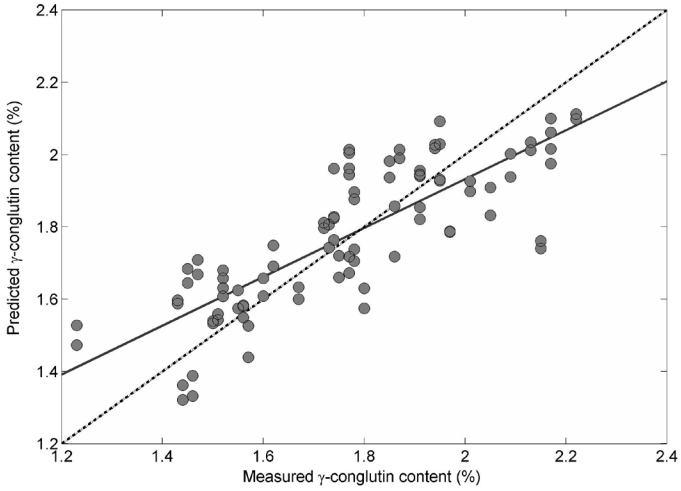

Fig. 1: Scatter plot of measured vs. predicted seed γ-conglutin content (in % w/w) for 46 genotype-environment combinations. Prediction based on a Near-Infrared Reflectance spectroscopy calibration curve, using cross-validations (continuous line = regression line; broken line = 1:1 line)

different cropping environments. Other seed quality traits of white lupin displayed less favourable responses, for example tocopherol content, whose genetic improvement proved hindered by high genotype × environment interaction (ANNICCHIARICO et al., 2014). The scope for selecting for higher seed γ-conglutin content is reinforced by the possibility to achieve values of γ-conglutin proportion on total protein that exceed the 4-5% range reported in literature (DURANTI et al., 2008), as in the case of the genotypes 7-50 and Lucky (5.3%; Tab. 2).

Our NIRS calibration results, although preliminary, suggest that NIRS-based predictions for seed γ-conglutin content are not sufficiently accurate to substitute completely for chemical analyses. However, NIRS data could be exploited in preliminary selection stages for higher seed γ-conglutin content, performing the costly chemical analysis for this trait only on breeding lines promoted to final selection stages. We verified by earlier work that NIRS calibration for seed γ-conglutin content based on spectra recorded from whole grain samples, which are exploitable also for selection of individual seeds, are characterized by too modest predicting ability to be of practical use ($R^2 = 0.27$; FERRARI et al., 2015). NIRS prediction based on ground seed samples can be used in breeding programs to select genotypes on the basis of seed γ-conglutin content of their bulked progeny seed.

The greater dependency of seed γ-conglutin content of lupin cultivars from γ-conglutin proportion on total protein than from seed protein content, which emerged from regression analyses, has practical implications for breeding programs. It suggests that selection for higher protein content, which could reliably be performed by NIRS measurements, may have limited value for increasing seed γ-conglutin content.

Our results show that high γ-conglutin production per unit area, which is the ultimate target for industrial production of γ-conglutin, is largely affected also by high grain yielding ability of the genotypes. Therefore, breeding strategies aimed to improve crop yields, e.g., by exploiting innovative plant types, stress tolerance, global genetic resources and adaptation to specific climatic conditions (HUYGHE, 1997; ANNICCHIARICO et al., 2010), remain of utmost importance even when targeting higher yield of this protein fraction.

The current information on environmental variation for γ-conglutin traits is limited. Its results for the season 2013-14 suggest that a favourable environment may increase the crop production of γ-conglutin not only via higher grain yield but also via higher seed γ-conglutin content that derived, in this case, from higher seed protein content. Results for three relatively favourable cropping environments in the seasons 2011-12 and 2012-13 failed to reveal distinct and/or consistent differences between the subcontinental-climate site of Lodi and the Mediterranean-climate site of Sanluri.

In conclusion, this study indicates that white lupin seed content of γ-conglutin can be enhanced through plant breeding. Selection costs can be reduced by adopting very few test sites because of limited genotype × environment interaction, and by relying on NIRS-based predictions in early stages of selection.

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