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Composition, environmental stability and potential of genetic improvement of fatty acids of *Lupinus angustifolius*

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Summary

In the last decades procedures for obtaining protein isolates and concentrates derived from narrow-leafed lupins (*L. angustifolius*) for human nutrition have been developed. Since this processes starts with defatting of seeds, lupin oil is obtained in large quantities. Therefore, 50 genotypes of *L. angustifolius* were analysed regarding the fatty acid (FA) composition of seed oil and the environmental stability of fatty acid contents in order to get information on the application of lupin oil in the food industry. The results revealed an n-3/n-6 poly unsaturated fatty acid ratio of 0.13. Furthermore, the seed oil of *L. angustifolius* contains rather high amounts of saturated FAs (22 %). Significant genotypic differences and a high heritability (h^2>85 %) for the content of all fatty acids are suggesting that the potential for genetic improvement of fatty acid composition by breeding is given. However, coefficients of variation below 10 % for all considered traits point out that a rapid improvement in seed oil quality will be hindered by the narrow genetic base of the breeding material tested.

Introduction

Lupins belong to the legume family and have been used as a food for over 3000 years in the Mediterranean (GLADSTONES et al., 1998). Since their introduction to Northern Europe in the 18th century, lupins are locally grown in Germany (HONDELMANN, 1984). But because of their high seed alkaloid content they did not gain remarkable agricultural importance. With the discovery of sweet mutants of *L. luteus* (yellow lupin), *L. albus* (white lupin) and *L. angustifolius* (narrow-leafed lupin) at the beginning of the 1920th, lupins became fully accessible as feed and food crop (SENGBUSCH, 1938). However, lupin cultivation remained far behind other crop plants due to limited yield and breeding progress. Just in recent years lupins have been rediscovered to be used in the food industry. Among the local grain legumes, lupins show a quite unique seed composition, which make them well suited for a modern human nutrition. While having a high protein and oil content, lupin grain contains minimal starch, but is very rich in dietary fibre (SIPSAS, 2008).

Functional properties of lupin derived food ingredients, like lupin protein isolates, are very promising and processes for their recovery are described in several studies (AGUILERA and GARCIA, 1989; WAESCHE et al., 2001; SUSSMANN et al., 2013). While great attention has been paid on the characterization of lupin protein, up to now information available about the quality of lupin oil and its potential for genetic improvement is limited. Considerable amounts of lupin oil will be extracted and may be used in human nutrition, since most of the procedures for producing lupin protein isolate start with a defatting step (WAESCHE et al., 2001). In Germany, the species *L. angustifolius* is the most widely grown lupin species since the mid 1990s, due to their higher resistance against the fungal disease anthracnose (Colletotrichum lupini) (EICKMEYER, 2008). The oil content of German varieties of *L. angustifolius* is about 6 % (JANSEN and JUERGENS, 2008). A comparative study about the oil quality of the three sweet lupin species *L. albus*, *L. angustifolius* and *L. luteus* has been conducted by CHIOFALO et al. (2012). The study has identified narrow-leafed lupins to be the less suited lupin-species from a nutritional point of view, due to the highest content of saturated fatty acids and a low n-3/n-6 polyunsaturated fatty acid ratio. Up to now, to our knowledge no studies have been undertaken to get information on the environmental stability and the potential for genetic improvement of the fatty acid profile in narrow-leafed lupins. The evaluation of variation in fatty acid contents due to genotypic, environment and genotype x environment interaction (GE interaction) will be of interest for breeders and end users. Respective studies in which the influence of environment, genotype and GE interaction on the fatty acid composition was analysed, have been already carried out for *L. albus* seed (BOSCHIN et al., 2007; BOSCHIN et al., 2008). In this study we evaluate the oil content and the fatty acid composition of new German *L. angustifolius* breeding lines. We further assess the influence of environment, genotype and GE interaction on the variability in fatty acid contents and calculated heritabilities to estimate the potential for genetic improvement by breeding.

Materials and methods

Plant Material

Whole seeds of 50 genotypes of *L. angustifolius* L. were analysed for oil and fatty acid content. The seed samples were provided by the Saatzucht Steinach GmbH & Co KG (Bocksee, Germany) and comprised 42 advanced breeding lines and 8 German cultivars of *L. angustifolius* (Probor, Boregine, Borlu, Vitabor, Haagena, Sonate, Boruta, Haags Blaue). The two cultivars Haags Blaue and Boruta and 6 breeding lines belong to the restricted branching (determinate) type and the other ones belong to the branched (indeterminate) type.

Experimental design and characterization of environments

The trials were conducted at four different sites in Germany during three consecutive growing seasons (2010 - 2012). Three sites were located in Mecklenburg-Western Pomerania (Northern Germany), Bornhof (53°5'N, 12°9'O), Dratow (53°5'N, 12°8'O) and Groß Lüsewitz (54°1'N, 12°3'O) and one site was located in Bavaria (Southern Germany), Steinach (48°9'N, 12°6'O). The three sites located in Northern Germany were characterized by sub-acid soil pH, sandy earth (Bornhof) to loamy sand (Dratow, Groß Lüsewitz) and an annual rainfall from 558 mm (Bornhof), 559 mm (Dratow) to 683 mm (Groß Lüsewitz). Steinach located in Southern Germany is characterized by an acid soil pH of 6.0 but loamy earth and an annual rainfall of 784 mm. The experiments were conducted in a randomized complete block design with four replications and different plot sizes. In Steinach and Dratow the plot size was 4.5 m^2 (3.0 m x 1.5 m),

in Groß Lüsewitz 4.2 m^2 (2.8 m x 1.5 m) and in Bornhof 10.5 m^2 (7.0 m x 1.5 m). Seed density of branched types was 100 seeds per m^2 and of restricted branching types 120 seeds per m^2. For details confer to BEYER et al. (2015). For analyses of fatty acids mixed samples of replication 1 and 2 as well as 3 and 4, respectively, were used.

Chemical Analysis

Samples were ground to whole seed flour by using a break mill (Brabender SM3) first, followed by a falling number mill (Perten Laboratory Mill 3100), which generate flour passing through a sieve size of 0.8 mm. Each sample was analysed in duplicates.

Seed oil was extracted with petroleum ether (Sigma-Aldrich, Steinheim, Germany) using a Dionex ASE 200 accelerated solvent extractor. 5 g lupin flour was first dried in a moisture analyser (Ohaus-MB35) at 130 °C and then extracted for 45 minutes at 1500 psi at 130 °C and 3 static cycles at 130 °C and 3 static cycles of 11 ml flush volume each time. After evaporating the solvent, oil content was gravimetrically determined and expressed as weight percentage (%) on a dry matter basis.

Fatty acids were analysed as fatty acid methyl esters (FAME); 5 µl of the extracted oil was suspended in 1 ml tert-Butylmethylether/ Methanol mixture (1:1 v/v), 100 µl trimethyl sulfonium hydroxide solution (TMSH, 0.15 mol/l in methanol) were added, the mixture was shaken and transferred into a vial for GC analysis. The FAMEs were analysed by GC-FID (Agilent 6890) on HP-FFAP 25 m x 0.2 mm x 0.33 µm column with carrier gas H_2 and a flow rate of 1 ml/min in split mode (1:100); injection volume 2 µl, temperature of injector 280 °C and detector of 250 °C. The column temperature was programmed as follows: an initial temperature of 160 °C for 1 min, with increments of 15 °C/min up to 220 °C and increments of 30 °C/min up to the final temperature of 240 °C, which was hold for 2.83 min.

The single fatty acids were calibrated by using dilutions of AOCS Reference Mixes (Sigma-Aldrich Grain Fatty Acid Methyl Ester (47801) and Low Erucic Rapeseed Oil (O7756)) and the amounts were expressed per total fatty acid esters identified. Fatty acid (FA) groups were obtained by summing up the percentage of the appropriate FAs: total saturated FAs (total SFA): sum of C14:0 + C16:0 + C18:0 + C20:0 + C22:0 and C24:0; monounsaturated FAs (MUFA): C18:1n-7 + C18:1n-9 + C20:1; polyunsaturated FAs (PUFA): C18:2 + C18:3.

Statistical Analysis

All statistical analyses were performed using SAS software (SAS 9.3, Institute Inc., Cary NC, USA). Overall means of the oil and FAs were calculated and shown as least square means (lsmeans). Lsmeans and variance components for the evaluated traits were estimated using a linear mixed model, which fitted random effects by the restricted maximum likelyhood (REML) method. For calculation of lsmeans the genotype (g) was considered as fixed effect and locations (l), years (y) and the various interactions between g, l and y to be random. All factors were considered to be random in the model for estimation of the variance components and their standard errors. To evaluate the relationship among oil content and the various FAs, Pearsons correlation coefficients were calculated based on the lsmeans values of the 50 genotypes. Broad sense heritability (h^2) was calculated as:

$$h^2 = \sigma_g^2 / \sigma_p^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{gl}^2/l + \sigma_{gy}^2/y + \sigma_{gly}^2/ly + \sigma_e^2/rly)$$

were σ_g^2 is the genotypic variance (variance component for genotype), σ_p^2 the phenotypic variance, σ_{gl}^2 the variance component for genotype x location interaction, σ_{gy}^2 the variance component for genotype x year interaction, σ_{gly}^2 the variance component for genotype x location x year interaction, σ_e^2 the variance component for the error and l, y and r are number of locations, years and replications. Heritability is expressed on an entry mean basis using data from all genotypes, locations, seasons and replications. Principal component analysis (PCA) was carried out to characterize the influence of environment on fatty acid composition. The PCA analyses were calculated based on the lsmeans of the 12 experiments (l x y). Calculation of PCA was carried out using the JMP tool of the SAS software.

Results and discussion

In the seed oil of 50 varieties of *L. angustifolius* 6 saturated FAs and 5 unsaturated FAs were identified and quantified (Tab. 1). The major FAs found were linoleic acid (30.1 - 42.4%), oleic acid (28.8 - 39.0%) and palmitic acid (10.0 - 13.2%), accounting for more than 80% of the total FAs. The seed oil of *L. angustifolius* contains also significant amounts of linolenic acid, ranging between 4.2 and 6.0%. These results are in agreement with previous investigations of German narrow-leafed lupin varieties (JANSEN and JUERGENS, 2008) and also values reported by CHIOFALO et al. (2012), who investigated the fatty acid profile of three released cultivars of *L. angustifolius*. From a nutritional point of view, public health institutes, such as German Nutrition Society (DGE), or the Food and

Tab. 1: Estimates of lsmeans, standard deviation (SD), range and relative standard deviation (CV) of oil content (%) and single FAs (% of total FAs) of 50 genotypes of *L. angustifolius,* grown in 12 field trials in Germany

Fatty acid	Overall lsmean ± SD	Range (%)	CV	P-value of ANOVA[1]
oil content[2]	6.3 ± 0.4	5.7 – 7.2	6.1	***
SFA				
C14:0 (Myristic acid)	0.3 ± 0.0	0.2 – 0.3	9.2	***
C16:0 (Palmitic acid)	11.4 ± 0.7	10.0 – 13.2	6.4	***
C18:0 (Stearic acid)	6.7 ± 0.7	5.3 – 8.6	9.7	***
C20:0 (Arachidic acid)	1.0 ± 0.1	0.8 – 1.2	8.4	***
C22:0 (Behenic acid)	2.0 ± 0.1	1.6 – 2.3	6.6	***
C24:0 (Lignoceric acid)	0.6 ± 0.1	0.7 – 8.9	8.9	***
total SFA	21.8			
MUFA				
C18:1n-9 (Oleic acid)	33.8 ± 2.1	28.8 – 39.0	6.3	***
C18:1n-7 (Vaccenic acid)	0.8 ± 0.1	0.7 – 1.0	10.5	***
C20:1 (Gadoleic acid)	0.3 ± 0.0	0.3 – 0.4	7.7	***
total MUFA	34.9			
PUFA				
C18:2n-6 (Linoleic acid)	37.8 ± 2.5	30.1 – 42.4	6.7	***
C18:3n-3 (Linolenic acid)	5.1 ± 0.4	4.2 – 6.0	8.1	***
total PUFA	42.9			
n-3/n-6 PUFA	0.13			

[1] for genotypic differences, with *** $P \leq 0.001$
[2] cf BEYER et al., 2015

Agriculture Organization (FAO) have given recommendations for the daily intake of specific FAs with dietary relevance. Saturated FAs (SFA) should account for less than 10 %, monounsaturated FAs (MUFA) for more than 13 % and polyunsaturated FAs (PUFA) for 7 - 10 % of the overall energy intake (Deutsche Gesellschaft für Ernährung, 2000). Special emphasis is laid on mutual proportion of n-3/n-6 PUFA in the diet since our modern nutrition depends increasingly on cereals, having an unfavourable low α-linolenic acid content (n-3 FA) and excessive amounts of linoleic acid (n-6 FA) (Simopoulos et al., 2002). A diet containing a ratio of 0.2 of n-3/n-6 FAs is recommended by the German Health Authority (Deutsche Gesellschaft für Ernährung, 2000). In this respect, our results of total saturated fatty acid content with 21.8 % in *L. angustifolius* revealed a high proportion in comparison to other vegetable oils, like soybean oil (15.7 %), sunflower oil (12.8 %), or rapeseed oil with contents of 8.0 % SFA (Dubois et al., 2007). The analysed average n-3/n-6 PUFA ratio of 0.13 in the breeding material is below the recommended threshold of 0.2, but it is comparable with the ratio of soybean oil (0.15) and distinctly higher than that of sunflower oil, which is below 0.01 (Dubois et al., 2007). ANOVA revealed significant differences between genotypes in fatty acid contents ($P < 0.001$), whereas the coefficient of variation below 10 % for almost all FAs indicate limited genotypic variation within the breeding material tested (Tab. 1). Therefore, breeding for a better fatty acid composition might be restricted by the narrow genetic variation in these traits.

Variance components of environments (l, y and l x y), genotype (g) and genotype x environment interactions (g x l, g x y and g x l x y) were estimated for the most abundant FAs via REML analysis and are shown in Tab. 2. In general, the fatty acid contents were mostly influenced by the growing season (y), except palmitic acid. This indicates that fatty acid composition varies more due to changing weather conditions than to locations. Since climatic conditions cannot be controlled, it will be difficult to make predictions about fatty acid composition of lupin seed oil in advance. The genotypic variance component is large in comparison to the sum of GE interaction variance components for all considered FAs, indicating that the ranking of genotypes is fairly stable across environments. This also leads to high values of broad sense heritability on an entry mean basis ($h^2 > 0.85$ for all traits), which facilitates genetic progress in improving fatty acid composition of lupin seed oil. Our findings about the genotypic and environmental influences on fatty acid composition of *L. angustifolius* seed is in accordance with reports of Boschin et al. (2008), who investigated the effect of genotype and environment on fatty acid composition of *L. albus* seed. Boschin et al. (2008) also reported high broad sense heritability for fatty acid content depending on a large ratio of genotypic to GE interaction variance components.

Tab. 2: Variance component estimates of genotype (g), locations (l), years (y), error (e) and genotype x environment interactions, as well as heritability estimates on an entry mean basis of FAs of *L. angustifolius* grown in 12 field trails in Germany

Source	C16:0	C18:0	C18:1	C18:2	C18:3
g	0.51***	0.4***	4.0***	5.5***	0.15***
l	0.05	0.1	6.3	7.7	0.04
y	0.00	2.0	10.4	17.8	1.52
l x y	0.26	0.2	1.0	1.1	0.20*
g x l	0.03***	0.03*	0.5***	0.3***	0.03***
g x y	0.03***	0.1***	0.5***	0.7***	0.01***
g x l x y	0.07***	0.2***	1.4***	1.6***	0.06***
e	0.08***	0.1***	0.6***	1.0***	0.03***
h^2 (%)	95.1	85.4	90.8	92.3	89.4

* Significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$

Pearson's correlation estimates among oil and the five main FAs are given in Tab. 3. Oil content was significantly inversely related to the palmitic acid and linolenic acid and significantly positively correlated to the stearic acid content. The negative association between linolenic acid and fat content has also been stated by Uzun et al. (2007), whereas Chiofalo et al. (2012) reported a significant positive correlation between oil content and n-3 PUFAs in lupin seed. In our results, oleic acid has a strong negative correlation with linoleic acid. An inverse association between these two FAs has already been observed in lupin seed (Uzun et al., 2007; Chiofalo et al., 2012) and is well documented in other oilseed crops such as soybean (Bachlava et al., 2008), sesame (Brar, 1982) or peanut (Andersen et al., 1998). In this study oleic acid showed a negative relation to linolenic acid and a positive correlation to stearic acid. A negative relationship was also observed between stearic acid and linoleic acid as well as linolenic acid (Tab. 3). A negative association between SFA and n-6/n-3 PUFAs was also reported by Chiofalo et al. (2012), but not on a significant level. The observations suggest that selection for genotypes with high oil content and high oil quality at the same time might be difficult due to the strong negative correlation between oil content and n-3 PUFAs.

Tab. 3: Correlations between oil content and FAs of *L. angustifolius* genotypes

	Oil	C16:0	C18:0	C18:1	C18:2
C16:0	- 0.50 ***				
C18:0	0.39 **	- 0.26			
C18:1	0.24	- 0.23	0.63 ***		
C18:2	- 0.01	- 0.12	- 0.68 ***	- 0.91 ***	
C18:3	- 0.72 ***	0.24	- 0.35 **	- 0.38 **	0.18

** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$

Principal Component Analysis (PCA) was conducted based on the five main FAs to characterize the influence of environment on the fatty acid composition (Fig. 1). The first two principal components account for 94.3 % of the total variation. PCA axis 1 was dominated by the strong negative association between oleic acid and the polyunsaturated FAs, whereas PCA axis 2 based mostly on palmitic acid and its slightly negative association to the unsaturated FAs. Fig. 1 revealed that environments are predominantly grouped according to the growing season and not according to locations, which is in accordance with findings of variance component analysis (Tab. 2). Growing season 2010 coused high amounts of stearic and oleic acid, whereas the years 2011 and 2012 resulted in high amounts of polyunsaturated FAs in lupin seed oil.

From a dietary point of view, seed oil of *L. angustifolius* could be improved concerning SFA content as well as n-3/n-6 PUFA ratio. Although significant differences between genotypes for all FAs are present, the genotypic range is quite limited. Heritability estimates on an entry mean basis are high for all considered FAs, but effective selection for better fatty acid profile will be hindered by the narrow genetic base of the breeding material tested. Therefore, broadening of the genetic base of *L. angustifolius* with respect to the fatty acid composition but also additional traits, e.g. protein content (Beyer et al. 2015) is needed.

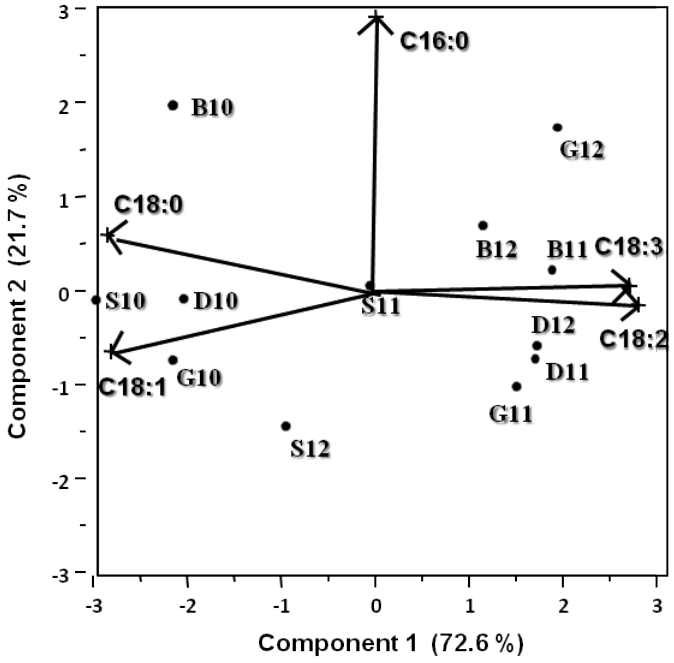

Fig. 1: Principal component biplot with scores represented by 12 trials and loadings represented by 5 FAs. The dots of the scores are labeled as follows: Bornhof (B), Dratow (D), Steinach (S) and Groß Lüsewitz (G) in the years 2010 (10), 2011 (11) and 2012 (12).

Tab. 4: Eigenvectors and Eigenvalues of the first two principal components for the 5 main FAs in seed oil of 50 breeding lines of *L. angustifolius*

Variable	Eigenvectors	
	PC1	PC2
C16:0 (%)	0.009	0.957
C18:0 (%)	-0.503	0.196
C18:1 (%)	-0.498	-0.209
C18:2 (%)	0.508	-0.048
C18:3 (%)	0.490	0.021
Eigenvalue	3.6	1.1
Cumulative	0.73	0.22
Cumulative variance (%)	72.6	21.7

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