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**MEETING THE FERTILIZER DEMAND  
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PROCEEDINGS**

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# PHENOTYPIC INDICATORS TO IDENTIFY METHIONINE RICH EUROPEAN GRAIN-LEGUMES AND THE CORRELATION OF GRAIN METHIONINE CONTENTS WITH THE SULPHUR SUPPLY

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*Amino acid supplementation of livestock diets is not allowed in organic farming. To enhance the nutritional value of European grain legumes (*Pisum sativum* L., *Vicia faba* L., *Lupinus angustifolius* L.) by plant breeding, cheap and efficient selection techniques for higher grain methionine content are sought after. Phenotypic indicators (leaf colour, root length in ethionine stress) and S-content in seeds are promising indirect traits to identify methionine rich genotypes. Plants sufficiently supplied with methionine indeed showed darker leaves and developed longer roots when stressed with ethionine (a toxic chemical homologue to methionine). Site specific differences of S-amino acid contents were found in grains of the same genotype. The correlation between S-content and S-amino acid content of grains was strongly positive, whereas the correlation to methionine alone was looser. S-content can not be used as single selection criteria for methionine rich seeds. In connection with the root length in ethionine high seed S-contents hint to methionine rich seeds of *P. sativum* and *Lupinus angustifolius*.*

*Key words:* Organic farming, plant breeding, chlorophyll, S-content, radicle-length.

## INTRODUCTION

Home grown legumes are a valuable protein source for pure on-farm diets for livestock in organic farming. Whereas protein of *Glycine max* naturally has higher contents of methionine and also lysine typical European grain legumes (*Pisum sativum* L., *Vicia faba* L., *Lupinus angustifolius* L.) used in organic farms as component of animal food are relatively low in those amino acids. Due to the restrictions in addition of synthetic amino acids given in the rules of organic production [1], breeding strategies to improve natural contents of legume seeds are of interest [8]. In organic farming and even in the most European countries only classical

approaches of plant breeding techniques are allowed. Besides, up to know no attempt to vary amino acid composition by genetic engineering in legume-seeds without health risks has been reported [11]. Today's knowledge of gene loci that are responsible for the synthesis of single amino acids in the proteins [4] is not used in legume breeding programs, so other selection criteria have to be found to characterize plants with the desired qualities. Together with total S contents of plants phenotypic criteria to select traits with high methionine contents were successfully used in breeding concepts for *Glycine max* [6]. The applicability of the criteria "dark leaf green" and "radicle-length in ethionine", which is a chemical homologue to methionine and plant growth inhibitor, to identify methionine rich plants (et-test) was shown. Results of the application of those techniques to select methionine rich European grain legumes are described in the following. Since the metabolism of S containing components is dependant from the S supply of plants [5, 13] and several studies reveal its effect on protein composition and qualities [2, 3, 7, 10, 12] also site effects on the amino acid composition of the examined legumes species are described.

## MATERIALS AND METHODS

For pre-studies to verify the phenotypic effects of methionine enrichment in laboratory scale local cultivars of the species *L. angustifolius* (variety Boruta, Saatzucht Steinach GmbH), *P. sativum* (variety Hardy) and *V. faba* (variety Espresso, both Nordeutsche Pflanzenzucht) were used. Effects of methionine applications to the seeds on leaf colour in direction to a darker green were found in all species, the radicle length in ethionine (et-test) could be stabilized in *P. sativum* and *V. faba*. The methodology and results on these effects are given in detail in [14].

### S and amino acid analyses in a legume sample

To identify the bandwidth of the amino acid composition of the different legumes 46 varieties of *L. angustifolius*, 50 varieties of *P. sativum* and 25 varieties of *V. faba* were selected by the above mentioned plant breeding companies and analysed. Site influence on the amino acid contents was explored with 18 of these *V. faba* varieties which were cultivated parallel on two sites and supplied by the University of Göttingen. Site 2 suffered drought in the year of cultivation. Furthermore seeds of collected cultivars were planted and grown in Trenthorst, North Germany on a loamy site in 2008 to gain more knowledge about the influence of the variety on the S-content in the legume seeds. Seeds of single pods of the plants were harvested separately and seeds of one pod each were analysed on S contents.

The amino acid composition in the seeds was analysed by HPLC according to the European Union Guideline 98/64/EG. The S contents were analysed with an elemental analyser EUROEA 3000 according to Dumas. Before analyses legume seeds were grinded with a 0.12 mm sieve in a centrifugal mill Retsch ZM 200.

## Selection of plants from the et-Test

The et-test was performed at 200 seedlings from M1 and M2 seeds from parents which were chemically mutagenized in Ethylmethanesulfonate (EMS). 25 seedlings with medium and 25 seedlings with higher root length were selected and brought to harvest in greenhouses. The seeds were screened for amino acid and seed S-contents. Additionally the chlorophyll content of the upper three fully developed leaves of the plants at flowering was determined [9].

## RESULTS AND DISCUSSION

### Bandwidth of selected amino acid composition

The highest concentrations of S-amino acids in relation to the protein [g/16gN] in the legume sample were between 12 % (methionine *V. faba*) and 25 % (methionine *L. angustifolius*) over the sample mean. The highest absolute methionine concentrations in [g/kg] laid between 16 and 26 % over the mean values of the different species. Maximum values of N- and S-contents in the seeds laid between 12 % (*V. faba*) and 67 % (*P. sativum*) for N and 32 % (*P. sativum*) to 40 % (*V. faba*) for S over the sample mean of the respective species (Table 1).

Table 1

Mean concentrations of N, S and different amino-acids of selected local legume species from Western Europe, cultivars from local seed breeders

Amino acids (mean, range)		<i>P. sativum</i>	<i>V. faba</i>	<i>L. angustifolius</i>
<i>n</i>		49	46	46
N	[%]	3.7 (2.9–6.2)	3.5 (3.3–3.9)	4.9 (3.9–5.9)
S	[%]	0.19 (0.13–0.25)	0.17 (0.13–0.23)	0.29 (0.21–0.38)
Cysteine		1.4 (1–1.6)	1.1 (0.9–1.2)	1.5 (1.1–1.8)
Methionine	[g/16gN]	1 (0.8–1.1)	0.7 (0.6–0.8)	0.7 (0.5–0.9)
Lysine		7.3 (6.3–7.9)	6.3 (5.7–6.9)	4.8 (4.2–5.4)
Cysteine		3.2 (2.3–3.7)	3 (2.3–3.5)	4.3 (3.5–5.4)
Methionine	[g/kg]	2.3 (1.9–2.8)	1.9 (1.7–2.3)	2 (1.8–2.4)
Lysine		17 (14.2–20.8)	17.2 (14.5–19.2)	14.4 (12.4–16.8)

### Site and variety effects

Looking at the effect of different growing conditions it is obvious that site and climatic conditions influenced the quality of the legumes. Under drought stress the N- and S-contents of *V. faba* seeds and consequently the absolute content of S-amino acids and also of lysine increased. In the contrary the protein quality was negatively influenced. Methionine in the protein (g/16gN) decreased (Table 2) and cysteine contents remained constant. Also lysine contents and the contents of most other amino acids responded in the same way to the stress conditions.

Table 2

Impact of different environmental conditions on the amino acid composition of *V. faba* seeds. Difference of drought stress site to control site is displayed in percent; n = 18 varieties per location.

	Control	Drought stress	Aberration
N [%]	3.88	4.38	12.9 ***
S [%]	0.15	0.17	18.9 ***
Crude protein [g/kg]	254 ± 18	286 ± 14	12.6 ***
<b>Amino acids [g/kg dry matter]</b>			
Cys	2.7 ± 0.2	3.1 ± 0.2	14.8 ***
Met	1.8 ± 0.1	2.0 ± 0.1	11.1 ***
Cys+Met	4.6 ± 0.3	5.1 ± 0.3	10.9 ***
Lys	16.4 ± 0.9	17.7 ± 0.5	7.9 ***
	<b>[g/16g N]</b>		
Cys	1.08 ± 0.09	1.1 ± 0.07	1.3 n.s.
Met	0.72 ± 0.04	0.7 ± 0.03	-2.8 *
Cys+Met	1.8 ± 0.12	1.8 ± 0.09	-0.3 n.s.
Lys	6.48 ± 0.23	6.19 ± 0.28	-4.4 **

n.s.= non significant. n.s.: P>0.05 \* P=0.5-0.01 \*\* P=0.01-0.001\*\*\* P <0.001, Tukey Test resp. Mann-Whitney-U Test.

Between the seeds from all cultivars of the sample grown at one site in Trenthorst, 2008 and their parent seeds no systematic differences in S-contents could be found (Table 3), whereas *V. faba* seeds of the parent generation grown on different sites (Table 2) showed correlations in absolute seed-S-contents (r=0.74) as well an in absolute methionine contents (r=0.71).

Table 3

Differences in mean S-contents between parent seeds of a legume sample and its F1-seeds grown on one site in one year [g/kg]

	n	Parents S [%]	F1 seeds S [%]	t-test	Correlation r
<i>V. faba</i>	23	0.16	0.15	n.s.	-0.05 n.s.
<i>L. angustifolius</i>	47	0.29	0.28	n.s.	0.19 n.s.
<i>P. sativum</i>	40	0.19a	0.17b	**	0.30 n.s.

P>0.05 \* P=0.5-0.01 \*\* P=0.01-0.001\*\*\* P<0.001

Regarding the correlation of S-contents and S-amino acid contents in the seeds, it is obvious that the absolute cysteine content is mainly determining the S-content in legume seeds (Fig. 1). Methionine and cysteine contents in the other legume species in general behaved like this (Table 4). In *Vicia faba* under drought conditions the connection between S-amino acids and S contents in seeds was looser (Fig. 1). Further data analyses showed that correlations between absolute S-contents [g/kg] and S-amino acid contents in the seed protein [g/16gN] were not given, whereas the S content of seeds in seed protein [g/16gN] was correlated positively to the S-amino acids in the protein with coefficients between 0.31 and 0.59.



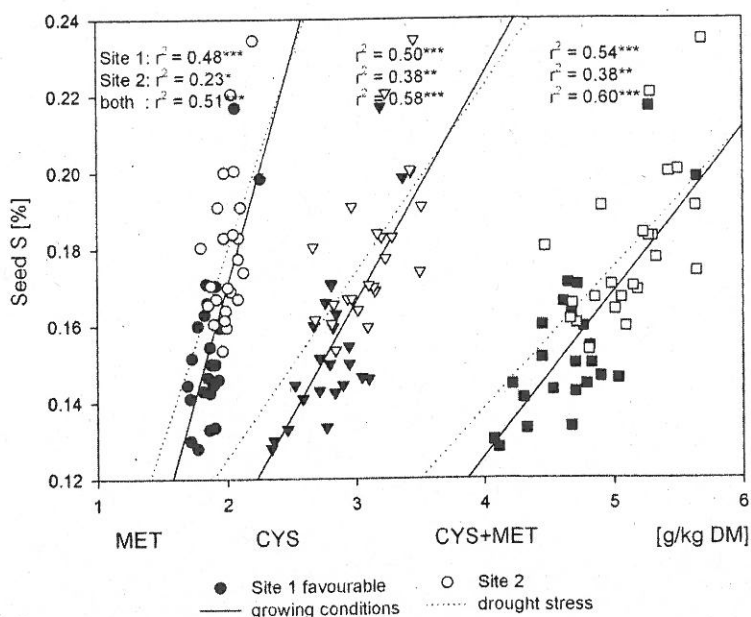


Figure 1. Relation of S-amino acid contents and S-contents in *V. faba* seeds from different varieties grown under different site and climatic conditions (n=23 each).

In *P. sativum* and *L. angustifolius* seeds of M1- or M2-plants of EMS treated parents from only one variety which were used in the et-test correlations of S-amino acids and total S-contents were comparable to the results found for different varieties. In *Vicia faba* no correlation was found between these parameters (Table 4).

Table 4

Correlation coefficients (r) of methionine and cysteine [g/kg] with S-contents [%] in *L. angustifolius* and *P. sativum* in a mixed sample of different varieties and origin

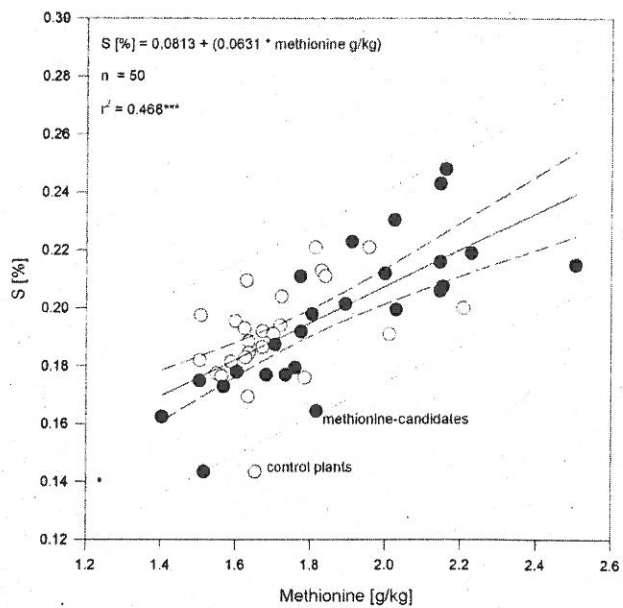
	n	Methionine	Cysteine	Cysteine + Methionine
<b>Different sites and varieties</b>				
S-content ( <i>V. faba</i> )	46	0.71***	0.76***	0.77***
( <i>L. angustifolius</i> )	46	n.s.	0.71***	0.66***
( <i>P. sativum</i> )	49	0.53***	0.64***	0.68***
<b>Equal sites and varieties</b>				
S-content ( <i>V. faba</i> )	50	n.s.	n.s.	n.s.
( <i>L. angustifolius</i> )	50	0.40**	0.65***	0.62***
( <i>P. sativum</i> )	50	0.69***	0.84***	0.80***

n.s.: p>0.05, \* p=0.05-0.01, \*\* p=0.01-0.001, \*\*\* p<0.001, <sup>1</sup>M1/Mx-plants of mutagenized arents (et-Test)

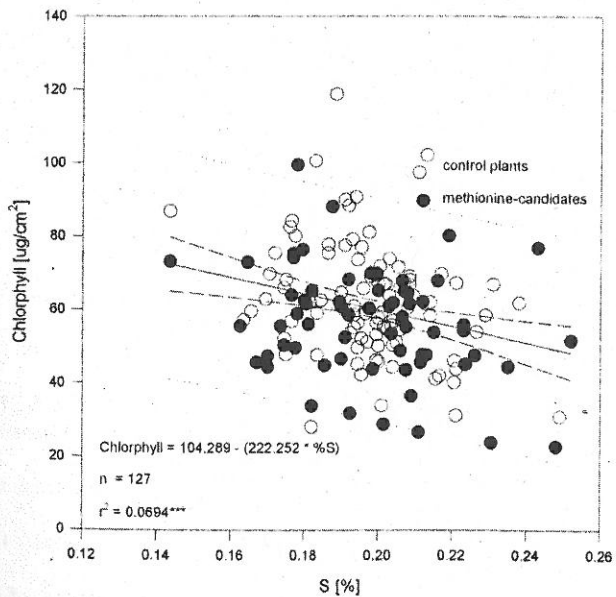
### Methionine, S and chlorophyll after selection from the et-test

The results of the selection of probable methionine rich *P. sativum* plants with the et-test are shown in Figure 2. Methionine rich plants could not be identified with the test on root length, but in the group of methionine-candidates

highest methionine contents were paired with high S-contents (Fig. 2A). Comparable trends were found in *L. angustifolius*. The leaf chlorophyll content of *P. sativum* showed neither correlation with the seed-methionine (not shown) nor the seed-S (Fig. 2B).



A



B

Figure 2 A. Relation of methionine contents and S-contents of seeds resp; B. Relation of the chlorophyll contents in younger leaves of *P. sativum* from parent plants with different radicle length in methionine (methionine candidates: long radicles in methionine, control plants mean radicle length in methionine).

## CONCLUSIONS

Aim of the study was to find out if plants with methionine rich seeds can be identified by cheap phenotypic and chemical indicators. S-content in seeds, leaf chlorophyll content and radicle-length in ethionine of growing plants were evaluated as selection criteria.

1. Regarding the heterogeneity of S-amino acid contents in different legume seeds – methionine contents from 12–25 % over the sample mean were found – a simple selection of e.g. methionine rich genotypes could enhance the supply in organic feed rations.
2. In the sample of selected legumes a positive correlation of the absolute S-amino acids with the S-content of seeds with correlation coefficients reaching the range of 60–80% was found whereas the correlation of methionine contents and S-contents was more indifferent.
3. High correlation coefficients for absolute and relative methionine contents between *V. faba* seeds of the same variety and generation from different sites hint for genetically based differences. But the missing correlation between the seed S-contents of parent seeds and their second generation grown at one site indicate to higher heterogeneity.
4. But it has to be considered that correlation of the determined S-content with the content of S-amino-acids in seeds was not found to be good enough to act as single criterion to identify methionine rich cultivars in the et-test. Only the combination of the criteria root length in EMS and S-content of seeds gave first consistent hints which plants should be selected for further breeding steps.
5. In awareness of the heterogeneity of the described indirect criteria also cheaper analysis procedures for direct analyses of amino acids like NIR should be introduced for an effective and definite screening on methionine rich legume seeds.

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