Phenotypical indicators for the selection of methionine enriched local legumes in plant breeding

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Abstract

One of the major problems in feeding organic pigs and poultry with 100 % organic feedstuff is the insufficient methionine (Met) content in the protein of local European grain legumes. The aim of the presented work is to find practicable and cheap indicators to select breeding candidates for growing legume cultivars with high seed-Met content under field conditions. In addition to direct Met determination a successful method for the phenotypical identification of Met enriched soybeans (*Glycine max*) developed by Imsande (2001) is assigned to peas (*Pisum sativum* (L.)), faba-beans (*Vicia faba* (L.)) and lupines (*Lupinus angustifolius* (L.)). Indicators were tested on a laboratory scale: chlorophyll content of the plant leaves and the root length in presence of an ethionine solution (0.75 mM) as competitor for Met. First results obtained from plants grown under Met-supply indicate a positive correlation between high Met and chlorophyll contents for *L. angustifolius* and *V. faba*. Plants treated with 1 mM Met show increased chlorophyll contents up to 59 % (*L. angustifolius*) and 34 % (*V. faba*) compared to untreated plants. In a second test, the phytotoxic effect of ethionine, a derivative of Met, is used to screen for Met-rich plants. The toxic effect is lessened with increasing Met concentrations. Seeds were soaked over night in a 1 mM Met solution developed up to 33 % (*P. sativum*) respectively 18 % (*V. faba*) longer roots. After the first successful steps to verify the methods in the laboratory, these detection methods for Met rich plants are currently transferred to practical use in the next phase of the project.

Keywords: legumes, methionine, plant breeding, organic farming

Zusammenfassung

Phänotypische Merkmale für die Selektion heimischer Leguminosen auf Methioninreichtum in der Pflanzenzüchtung

Ein Problem der Fütterung von Schweinen und Geflügel mit 100 % Futtermitteln aus Ökologischen Landbau sind die unzureichenden Methionin-(Met)-Gehalte im Protein europäischer Körnerleguminosen. Ziel der hier präsentierten Arbeiten ist es, neue Leguminosen Kultivare mit hohem Met-Gehalt im Samenprotein zu identifizieren. Neben der direkten Analyse der Aminosäuregehalte eines Zuchtsortiments der Pflanzenarten wird dazu die Übertragbarkeit der Methoden eines bei Soja (*Glycine max*) erfolgreich angewandten Verfahrens zur phänotypischen Selektion Met-reicher Pflanzen nach Imsande (2001) an Erbsen (*Pisum sativum* (L.)), Ackerbohnen (*Vicia faba* (L.)) and Lupinen (*Lupinus angustifolius* (L.)) erprobt. Als Indikatoren für Pflanzen mit hohem Metgehalt dienten der Chlorophyllgehalt der Blätter und das Wurzelwachstum von Keimlingen in einer Ethionin Lösung (0,75 mM). Erste Ergebnisse im Labormaßstab an unter Met-Zugabe aufgezogenen Pflanzen zeigten bei *L. angustifolius* and *V. faba* eine positive Korrelation zwischen der Met-Versorgung und den Chlorophyllgehalten. Met-reiche Pflanzen zeigten im Vergleich zu den unbehandelten Pflanzen um bis zu 59 % (*L. angustifolius*) bzw. bis zu 34 % (*V. faba*) erhöhte Chlorophyllgehalte in den Blättern. In einem zweiten Ansatz wurde der phytotoxische Effekt von Ethionin, einem chemischen Derivat zu Met, zum Screening auf Met-reiche Pflanzen genutzt. Der phytotoxische Effekt wird durch ansteigende Met-Konzentrationen gemindert. In einer 1 mM Met Lösung inkubierte Samen bildeten in Ethionin Lösung bis zu 33 % (*P. sativum*) bzw. bis zu 18 % (*V. faba*) längere Wurzeln aus. Die Erfolg versprechenden Ansätze zur Selektion Met-reicher Pflanzen mit direkt mit Met behandelten Pflanzen werden derzeit an Feldpopulationen erprobt.

Schlüsselworte: Leguminosen, Methionin, Pflanzenzucht, Ökologischer Landbau

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Introduction

One of the major problems in feeding monogastric animals (organic poultry and pigs) with 100 % organic components in organic farming is the low content of essential amino acids (Met, Lysin) of the feed components. Low protein qualities have to be compensated either by protein excess with negative consequences for the environment and animal performance or by adding high quality protein sources like corn gluten that are not sufficiently available in organic quality. The addition of synthetic amino acids is not permitted in organic farming (EU) or is permitted for a limited time only (US) (Fanatico et al., 2007; Fanatico, 2008; Sundrum et al., 2005; Weissmann et al., 2005; Zollitsch et al., 2000, 2003 and 2007). Grain legumes represent a relatively high quality feedstuff, but a major drawback is that their seeds contain comparatively low amounts of sulphur-containing amino acids (Abel et al., 2002). On the other hand, it has been shown that Met and cysteine contents range widely among the cultivars of each species. Between lupine cultivars (*L. angustifolius*), for example, the Met bound in proteins varies up to 25 % (Jansen et al., 2006). Therefore one aim of the project is to analyze the bandwidth of amino acids in different cultivars of the three legume species. This is fundamental for breeding attempts in order to select promising cultivars. Additionally the genetic variability in the species is thereby investigated. Of each species 50 cultivars (partly also inbred lines) are analyzed.

Previous breeding attempts for legumes were mainly addressed to whole protein yield, stability and pest resistance (Steinberger, 2002). In this work a successful method by Imsande (2001) for the selection of Met enriched soybeans (*Glycine max*) by phenotypical indicators is assigned to legumes of moderate climatic conditions (*P. sativum*, *V. faba* and *L. angustifolius*). The results of the first project phase done in laboratory scale are presented here. The usability of two phenotypical indicators for the selection of young plants with elevated Met contents described for *Glycine max* should be verified. Firstly, an increased leaf greening induced by Met nutrition is investigated visually and by measured leaf chlorophyll contents. Secondly, the root length growth in presence of the phytotoxic Met analogue ethionine was analyzed. In a next step of research these tests will be transferred to field scale and the practicability of selection will be tested with known Met rich cultivars. The screening procedure will be applied also in populations from chemically mutagenized seeds to check legume cultivars with high seed-Met content for the use as advanced feed component in organic farming.

Material and methods

Experiments were conducted with local cultivars of the species *L. angustifolius (*variety Boruta, Saatzucht Steinach GmbH), *P. sativum* (variety Hardy, Norddeutsche Pflanzenzucht (NPZ)) and *V. faba* (variety Espresso, NPZ). Plants were grown in vermiculite under constant conditions in a climate chamber at 23 °C under a 12h/12h day/night regime. To study the Met effect, plants were treated with three different concentrations of Met (0, 0.1 mM and 1 mM) in an aqueous solution. Chlorophyll measurements were performed according to a method developed by Lichtenthaler (2001). The plant leaf area was previously determined with the "Leaf Area Measurement" software (www.shef.ac.uk/~nuocpe/).

Fluorescence measurements were conducted with the Junior Pam (Heinz Walz GmbH). Plants were previously dark adapted for 20 minutes. Thereby a change in the photosynthetic performance of plants can be investigated (Baker, 2008).

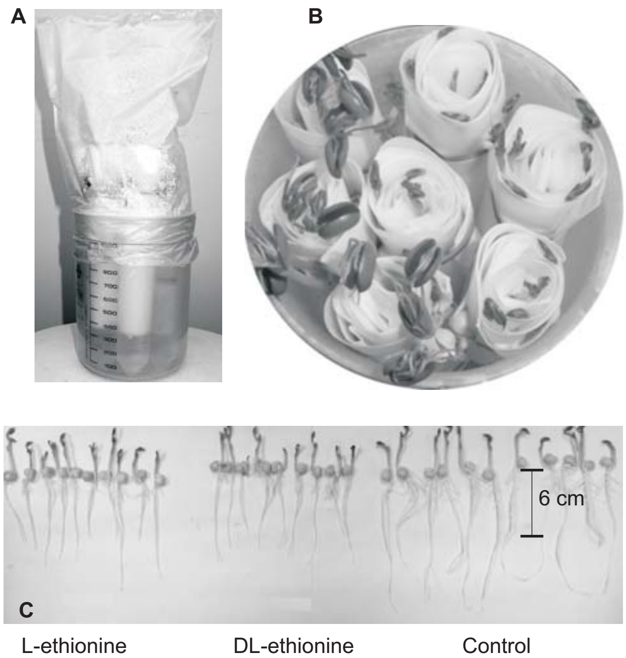

Figure 1:

Ethionine test composition with *P. sativum* and *L. angustifolius* in a smaller scale.

A: Beaker covered with a plastic bag for the storage of seed during germination test

B: Top view into a beaker containing germination paper with seeds

C: Determination of the root length in presence of 0.75 mM L- or DL-ethionine, respectively.

The ethionine test was performed with seeds of each plant species. Seed surfaces were sterilized with sodium hypochlorite (6.5 %). Afterwards the seeds were thoroughly washed with distilled water and soaked in a L-Met solution (1 mM) for 10h. After drying, the seeds were spaced on filter paper and rolled to a column of 12.5 cm height. Fifteen of these columns were placed in a container of 13 cm diameter (Figure 1).

450 ml of either a 0.75 mM DL-ethionine solution or tap water (Control) was added and the containers were enclosed with plastic bags. All steps were performed under sterile conditions. The seeds were left for 7 days at 23° C for growing at a 12/12h day/night regime.

Gained data were statistically analyzed with SPSS software (student version 14.0). Data with normal distribution were tested by Anova. Data without normal distribution were analyzed with the Kruskal-Wallis test. Level of significance was always α=0.05.

The bandwidth of the amino acid composition in the seeds of cultivars from local seed breeders was analyzed by HPLC according to the European Union Guideline 98/64/EG.

Results

As an excerpt of the amino acid analysis the mean data and the range for the sulphur-amino acids and lysine are presented in Table 1. Seeds of *P. sativum* cultivars possess the highest Met content of all investigated legume species. *V. faba* and *L. angustifolius* seeds contain a similar amount of Met, whereby seeds of *V. faba* showed relatively low concentrations of sulphur containing amino acids.

Lysine is generally high concentrated in legume seeds. As well as Met, lysine is highest concentrated in seeds of *P. sativum.* The lowest lysine contents could be observed in *L. angustifolius*.

Table 1:

Mean concentrations of different amino-acids of selected local legume species from Western Europe (n=50, per species), cultivars from local seed breeders, HPLC method.

Amino acids (g/16g N)	*P. sativum*	*V. faba*	*V. faba* (winter)	*L. angustifolius*
Cysteine (mean)	1.5 ± 0.16	1.21 ± 0.1	1.2 ± 0.1	1.66 ± 0.2
Cysteine (range)	1.13 - 1.81	0.95 - 1.38	1.14 - 1.35	1.28 - 2.01
Methionine (mean)	1.09 ± 0.07	0.79 ± 0.05	0.78 ± 0.05	0.77 ± 0.07
Methionine (range)	0.91 - 1.23	0.64 - 0.88	0.73 - 0.85	0.62 - 0.93
Lysine (mean)	8.1 ± 0.38	7.05 ± 0.29	6.82 ± 0.15	5.36 ± 0.24
Lysine (range)	7.01 - 8.83	6.4 - 7.67	6.68 - 7.03	4.92 - 5.9

The legume species showed a different adaptation to the Met treatment. Plants supplied with methionine showed an altered phenotype towards a darker green leaf colour (Figure 2). While for *P. sativum* no significant increase of chlorophyll concentration could be measured in spite of a visible change in leaf colour, *L. angustifolius* and *V. faba* plants accumulated higher chlorophyll amounts due to Met nutrition (Figure 3). *L. angustifolius* plants treated with 1 mM Met showed a significant increase by 59 % of the chlorophyll content at 38 days after planting. At the other test intervals, the mean value of Met treated lupines was elevated but showed no significant difference to the control.

Figure 2:

Changes in leaf colour of *Pisum sativum* treated with different methionine concentrations in the nutrient solution. The five youngest leaves of one plant are displayed.

Significantly elevated chlorophyll contents were also observed in *V. faba* plants treated with 1 mM Met. At 27 days after planting an increase of 34 % was measured, while 6 days later the chlorophyll concentration was still elevated by 25 %. For all three plant species a decrease of the absolute chlorophyll content could be observed after a certain time of cultivation. This effect generally appeared about 33 days after planting.

Additionally fluorescence measurements were performed to investigate the effect of Met on the efficiency of the electron transport chain (results not presented here). In these experiments no evidence of an impact of Met nutrition on the photosynthetic efficiency could be provided. This indicates that no positive effect on the photosynthetic performance has been induced by Met application.

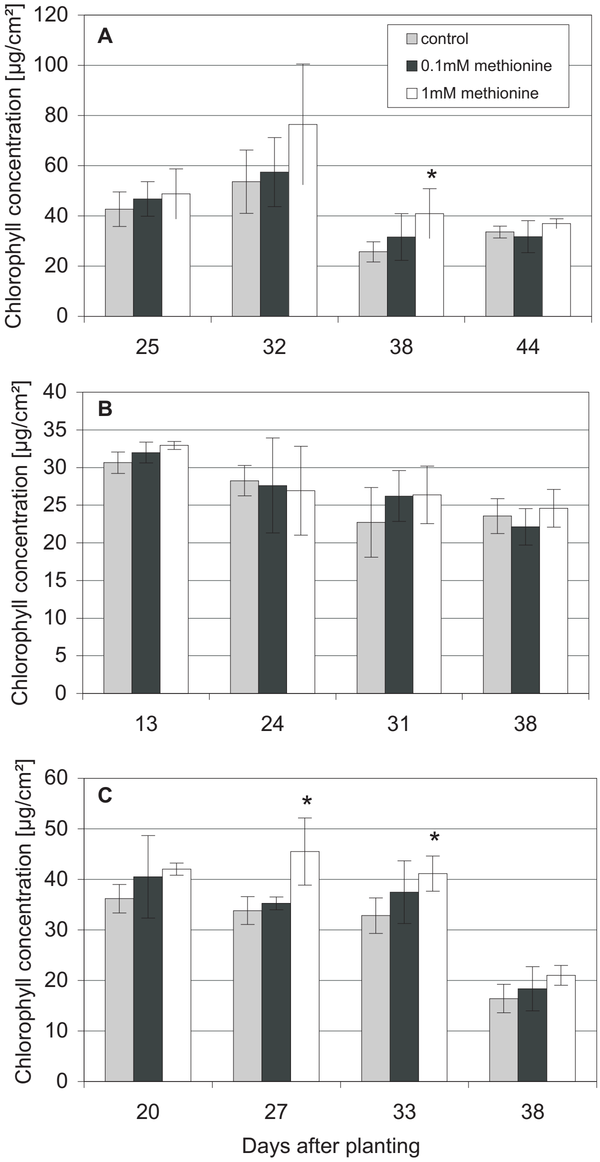

Figure 3:

Chlorophyll concentration of the youngest full expanded leaf of young legume plants. Asterisk indicates significant difference to the control of the same developmental stage (α =0.05). A: *L. angustifolius*; B: *P. sativum* C: *V. faba*; (n=6, per species)

The experiments performed with the Met analogue ethionine produced heterogeneous results with the plant species listed. *L. angustifolius* seedlings showed no reduction of the root length growth in ethionine solution (Figure 4A). On the contrary, the seedlings treated with ethionine developed longer roots than the control plants. However the difference was in no case significant. No difference could be observed between seeds pre-treated with Met and untreated plants.

P. sativum and *V. faba* seedlings behaved contrarily to the *L. angustiolius* findings in the experiment (Figure 4B+C). For both species ethionine treatment caused a significant root length reduction. *P. sativum* seedlings grown in ethionine had 38 % shorter roots. When the seeds were previously soaked in Met only, a root length reduction of 18 % occured. When comparing the two ethionine preparations, the Met treated seeds had significantly longer roots (Tukeys test ($p > 0.05$)).

Root growth of *V. faba* seedlings was as well inhibited by ethionine. Both ethionine preparations lead to significantly reduced root lengths. The pre-treatment with Met caused 20 % longer roots compared to untreated seeds in ethionine without being significant (Tukeys test ($p > 0.05$)).

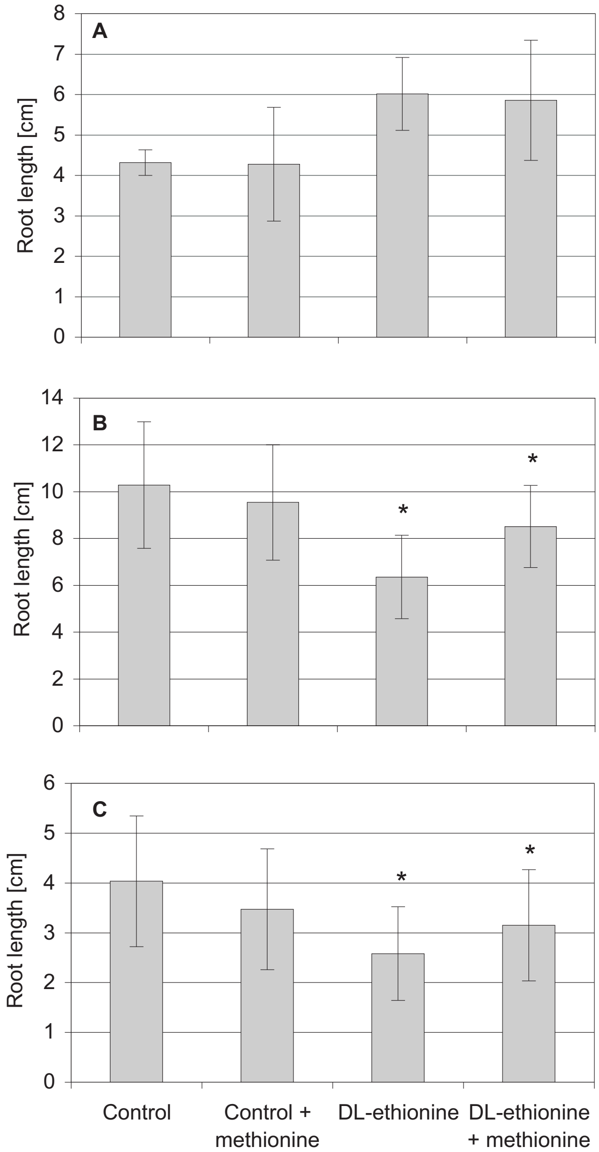

Figure 4:

Root length of seedlings of different legumes grown for 7 days in different solutions. Asterisk indicates significant difference to the control. Control=aqueous solution, seeds pre-treated with aqua dest.; methionine=aqueous solution and seeds pre-treated with 1 mM Met solution, DL-ethionine=0.75 mM ethionine solution seeds pre-treated with aqua dest; DL-ethionine + methionine=0.75 mM ethionine solution and seeds pre-treated with 1 mM Met solution. A: *L. angustifolius*; B: *P. sativum* C: *V. faba*; (n=10, per species)

Discussion and outlook

The experimental data indicate legume phenotypes which appear to be typical for Met-rich individuals. However the validity of the experiments seems to be dependent on the plant species. While the chlorophyll determination lead to promising results for *L. angustifolius* and *V. faba,* it did not correlate with the Met treatments for *P. sativum*. Also the visible changes in leaf green due to Met exposition need further evaluation. On the contrary the root length reduction in presence of ethionine provided the expected results for *P. sativum* and *V. faba* but not for *L. angustifolius*. Whether this is caused by the experimental design or the different plant physiology remains to be investigated. The obtained results confirmed that the experimental design created by Imsande (2001) is at least partly alienable to local legume species.

The analysis of sulphur-amino acids provides valuable knowledge for the selection of applicable cultivars. The high variability in the Met content between cultivars of up to 25 % claimed by Jansen et al. (2006) for *L. angustifolius* could be confirmed. In all three species the Met content differs by up to 25 % between the analyzed cultivars provided by the local seed breeders (Table 1). According to this data, cultivars with an already superior Met content and unchanged overall amino acid composition have been chosen for further experiments in the project. 2000 seeds of progency plants from each cultivar previously mutagenized with ethylmethanesulfonate (EMS) have undergone the ethionine test. From the developed seedlings 5 % with the longest and healthiest looking roots were selected as putative Met-rich individuals. These plants were brought to maturity for further breeding experiments. Alongside the chlorophyll content has been measured in a non destructive manner and compared to control plants. Harvested seeds from these plants will be tested for their amino acid composition by HPLC and compared to control plants.

Furthermore the investigation of the molecular background of the altered phenotypes which have got a high Met content will be performed. This shall be done by comparative proteome and transcriptome analysis of plants treated with methionine and Met-rich cultivars. Thereby the mechanism which induces changes in leaf chlorophyll concentration is of particular interest. Met is an important metabolite in the plants methylation reactions. Eighty percent of the free Met is converged into s-adenosyl-methionine (AdoMet) (Ravanel et al., 2004). AdoMet plays an important role in the chlorophyll-biosynthesis (Bollivar, 2006). This connection of the pathways is presumably a key point for the understanding of the altered phenotype and requires further investigation.

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