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EFFECT OF SELECTED DIVALENT CATIONS ON PROTEIN MOBILIZATION IN LENTIL (*LENS CULINARIS*) SPROUTS

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Abstract

The influence of calcium and magnesium on protein mobilization and non-protein nitrogen content in cotyledons of germinated lentil was studied.

Elicitation of metabolism in sprouts modifies the rate of protein mobilization and causes a subsequent increase in the non-protein nitrogen fraction in lentil cotyledons. Application of $MgCl_2$ proved to be the most beneficial, leading to the highest yield of non-protein nitrogen in cotyledons from 6-days-old lentil sprouts. Regardless of the type of elicitor, a decrease in the activity of proteolytic enzymes was observed in cotyledons from 3- and 4-day-old lentil sprouts. A significant correlation was found in sprouts elicited with $MgCl_2$ between the non-protein nitrogen content and activities of endo- and aminopeptidases.

The information provided by this study allows us to design conditions for lentil sprouting which will result in an enhanced level of potentially bioaccessible non-protein nitrogen (free amino acids and peptides).

Keywords: magnesium, calcium, protein mobilization, free amino acids, lentil, sprouting.

WPŁYW WYBRANYCH JONÓW DWUWARTOŚCIOWYCH NA MOBILIZACJĘ BIAŁEK W KIEŁKACH SOCZEWICY JADALNEJ (*LENS CULINARIS*)

Abstrakt

W pracy badano wpływ jonów wapnia i magnezu na szybkość mobilizacji białek i zawartość azotu niebiałkowego w kotyledonach kiełków soczewicy.

Elicytacja metabolizmu kiełków modyfikuje szybkość mobilizacji białek, powodując jednocześnie zwiększenie udziału frakcji azotu niebiałkowego w kotyledonach. Wykazano, że indukcja z użyciem roztworów $MgCl_2$ najefektywniej zwiększyła poziom azotu niebiałkowego w kotyledonach 6-dniowych kiełków. Bez względu na rodzaj elicytora, w kotyledonach 3- i 4-dniowych kiełków zaobserwowano spadek aktywności enzymów proteolitycznych. Dodatkowo, w kotyledonach indukowanych roztworami $MgCl_2$ określono wysoką dodatnią korelację między poziomem azotu niebiałkowego oraz aktywnością amino- i endoproteaz.

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Na podstawie wyników opracowano recepturę hodowli kiełków, które charakteryzują się zwiększonym poziomem potencjalnie biodostępnego azotu niebiałkowego (wolne aminokwasy i peptydy).

Słowa kluczowe: magnez, wapń, mobilizacja białek, wolne aminokwasy, soczewica, kiełkowanie.

INTRODUCTION

In recent years, food legumes have attracted much attention owing to their functional components and health promoting effects. The quality and potential bioactivity of low processed food such as sprouts is mainly determined by the quality of seeds and/or conditions of germination (Gawlik-Dziki et al. 2012, Świeca et al. 2012)

There are several reports about the effect of germination methods on the nutraceutical value of legumes, including, soybeans, mung beans or lentils (Zhao et al. 2005, Randhir, Shetty 2007). Most studies have been conducted using biotic and abiotic stresses and/or elicitors (Randhir Shetty 2007, Ghavidel Prakash 2007, Gawlik-Dziki et al. 2012). Elicitation of seedlings seems to be a useful technique used for improving the nutritional and nutraceutical potential of low-processed food.

Germination is one of the most common and effective processes for attaining better quality of legumes. During germination, the enhanced activity of proteases results in the immobilization of storage proteins (Muntz et al. 2001). Some proteases are known to require metals for their correct activity and/or are activated by metal ions (e.g. calcium- and calmodulin dependent proteases). Cytosolic calcium levels are drastically lowered during germination as a consequence of abscisic acid action (Khan 2010). There is some evidence that also Mg^{2+} may induce metabolic pathways involved in the mobilization of storage material and be responsible for the response to abiotic stress conditions (acts as an abiotic elicitor) (Shanker Venkateswarlu 2011). Additionally, calcium has been reported to inhibit Na^{+} uptake, thereby reducing its adverse effect on seed germination and stimulating the plant growth (Zehra 2012).

There is no information concerning the influence of calcium and magnesium on protein mobilization in germinated legumes, thus the objective of this study was to determine the influence of these cations on the content of protein and free amino acids at different germination stages of lentil. Additionally, an attempt was made to correlate the changes in free amino acid and protein fraction with the activity of proteases. The results obtained in this study could be valuable for designing conditions of lentil germination so as to develop functional food with an enhanced level of potentially bioaccessible non-protein nitrogen fraction (free amino acids and peptides).

MATERIAL AND METHODS

Seeds from the lentil cultivar Tina were purchased from the PNOS S.A. in Ozarów Mazowiecki, Poland. Seeds were sterilized in 1% (v/v) sodium hypochloride for 10 min, then drained and washed with distilled water until they reached neutral pH. They were placed in distilled water and soaked for 6 hours at 25°C. Seeds were dark germinated for 4 days in a growth chamber on Petri dishes (ϕ 125 mm) lined with absorbent paper. Seedlings were watered with 5 ml of Milli-Q water daily – control (GAWLIK-DZIKI, ŚWIECA 2011). For the experiments, 10 mmol dm^{-3} $CaCl_2$, 100 mmol dm^{-3} $CaCl_2$, 10 mmol dm^{-3} $MgCl_2$ and 100 mmol dm^{-3} $MgCl_2$ were selected as inductors. All solutions were freshly prepared before each application. For treatments, 2-day-old seedlings were watered daily with the tested solutions. Sprout samples were collected gently, the embryonic axis and seed coats were removed and cotyledons were frozen rapidly to be kept in polyethylene bags at -20°C until analyses. For each treatment, three replicates were prepared.

Analytical methods

Soluble protein and non-protein nitrogen (peptides and free amino acid rich fraction) were measured using the methodology described by PERIAGO et al. (1996). Approximately 25 cotyledons were extracted thrice with 25 ml of 0.02 mol dm^{-3} NaOH for 60 min and the three extracts were pooled. Insoluble material was removed by centrifugation at 8700 rcf for 20 min.

Soluble proteins were measured with the procedure described by LOWRY (1951), with bovine albumin as a standard. After that, the supernatant was mixed with 20 ml of 30% TCA and the mixture was stirred for 15 min at 4°C. Protein was removed by centrifugation at 8700 rcf for 15 min. Non -protein nitrogen was assayed using the ninhydrin method (SUNA 2006) and expressed as the leucine equivalent in mg g^{-1} f.m.

Extract preparation

Extracts for enzymatic assays were prepared from 25 cotyledons in each group. The cotyledons were homogenized in a mortar with 30 ml of 0.1 mol dm^{-3} TRIS HCl buffer pH 8 containing 150 mmol dm^{-3} NaCl and 10 mmol dm^{-3} β-ME. Samples were centrifuged at 8700 rcf for 20 min. The supernatants were used immediately to determine protease activity. All operations were carried out at 4°C.

Enzyme assays

Aminopeptidase activity was assayed using leucyl-*p*-nitroaniline (L-*p*NA) as substrate according to the method described by OGIWARA et al. (2005). Aminopeptidase activity was expressed in U per g of fresh weight (U g^{-1} f.m.).

One unit of enzyme activity is defined as an amount of enzyme required to release 1 μmol of pNA per in min. at the assay conditions.

The activity of endoprotease was assayed using hemoglobin as substrate and it was evaluated measuring peptide release by the increase in absorbance at 280 nm (ANSON 1938). Endoproteases were measures at 3 different pH using McIlvaine's buffers: acid (pH= 5.5), neutral (pH= 7) and alkaline (pH= 9). The endoprotease activity was expressed in U per g of fresh weight (U g^{-1}f.m.). One unit of enzyme activity is defined as an amount of enzyme catalyzing a change of 0.001 absorbance at the assay conditions.

Statistic

All experimental results were means ± S.D. of three parallel measurements. Two-way analysis of variance (Anova, the Tukey test) was used to compare groups within different elicitors. α values < 0.05 were regarded as a significant.

RESULTS AND DISSCUSION

The results regarding dormant seeds and sprouts before induction are summarized in Table 1. On germination, there was a statistically significant decrease in the soluble proteins content, which could be due to their being used as a source of energy by the sprouting process and/or intermediates for biosynthesis. This result agrees with an earlier report by RODRIGUEZ et al.

Table 1

Characteristics of dormant seeds and sprouts before elicitation

Specification	Dry seeds	Soaked	12-hours-old sprouts	24-hours-old sprouts
Proteins (mg g^{-1} f.m.)	132.35 ± 5.29^{b}	120.57 ± 4.82^{b}	106.45 ± 4.26^{a}	104.21 ± 4.17^{a}
Free amino acids and peptides rich-fraction (mg g^{-1} f.m.)	107.01 ± 8.49^{a}	126.36 ± 5.13^{b}	135.15 ± 5.41^{b}	172.18 ± 6.89^{c}
Aminopeptidase activity (U g^{-1} f.m.)	212.15 ± 7.56^{c}	128.29 ± 5.01^{b}	120.73 ± 4.86^{ab}	117.06 ± 3.78^{a}
Endoproteases activity at pH 5.5 (U g^{-1} f.m.)	79.49 ± 3.18^{b}	67.85 ± 2.71^{a}	93.23 ± 3.73^{c}	94.14 ± 3.77^{c}
Endoproteases activity at pH 7 (U g^{-1} f.m.)	104.78 ± 4.69^{b}	82.25 ± 2.87^{a}	101.74 ± 4.07^{b}	106.44 ± 4.26^{b}
Endoproteases activity at pH 9 (U g^{-1} f.m.)	111.65 ± 2.45^{d}	82.43 ± 3.12^{a}	99.91 ± 3.08^{b}	103.07 ± 4.12^{c}

Means followed by different letters are significantly different at $\alpha < 0.05$. Each value represents the mean of 3 measurements (±SE).

(2008). Simultaneously, an increase in the non-protein fraction (peptides and amino acids) was observed. This coincides with the findings of ROZAN et al. (2001) and KUO et al. (2004).

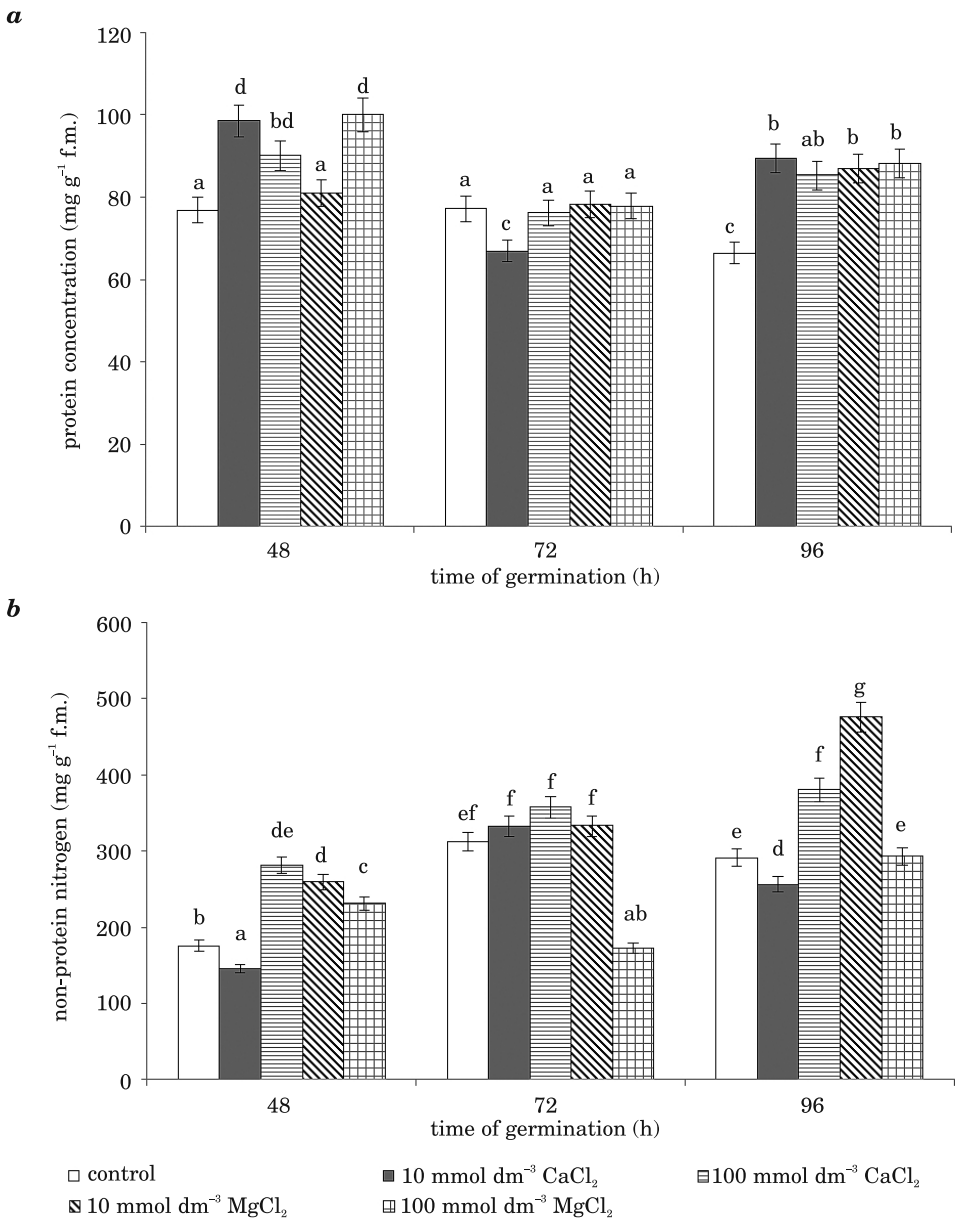

Fig. 1. Influence of $CaCl_2$ and $MgCl_2$ solutions on soluble protein (*a*) and non-protein nitrogen content (*b*)

Means followed by different letters are significantly different at $\alpha < 0.05$. Each value represents the mean of 3 measurements (±SE)

Table 2

Proteolytic activity in cotyledons of lentil sprouts elicited with $CaCl_2$ and $MgCl_2$ solutions

Specification	Day of germination (hours after induction)	Control	10 mmol dm^{-3} $CaCl_2$	100 mmol dm^{-3} $CaCl_2$	10 mmol dm^{-3} $MgCl_2$	100 mmol dm^{-3} $MgCl_2$
Aminopeptidase activity (U g^{-1} f.m.)	2 (24)	95.19 ± 3.81^{b}	87.84 ± 3.51^{ab}	101.84 ± 4.07^{bc}	92.13 ± 3.69^{b}	101.84 ± 4.07^{bc}
	3 (48)	111.07 ± 4.44^{bcd}	82.76 ± 3.31^{a}	97.89 ± 3.92^{b}	87.67 ± 3.51^{a}	97.89 ± 3.92^{b}
	4 (72)	105.39 ± 4.22^{bc}	108.39 ± 4.34^{c}	107.92 ± 4.32^{bc}	137.85 ± 5.51^{e}	124.03 ± 4.96^{de}
Endoprotease activity at pH 5.5 (U g^{-1} f.m.)	2 (24)	51.90 ± 2.08^{cd}	39.24 ± 1.57^{a}	46.05 ± 1.84^{b}	41.33 ± 1.65^{a}	46.05 ± 1.84^{b}
	3 (48)	60.67 ± 2.43^{d}	55.33 ± 2.21^{cd}	51.32 ± 2.05^{cd}	56.85 ± 2.27^{d}	51.32 ± 2.05^{c}
	4 (72)	88.82 ± 3.55^{e}	106.58 ± 4.26^{f}	92.86 ± 3.71^{e}	124.62 ± 4.98^{g}	106.72 ± 4.27^{f}
Endoprotease activity at pH 7 (U g^{-1} f.m.)	2 (24)	95.19 ± 3.81^{b}	87.84 ± 3.51^{ab}	101.84 ± 4.07^{bc}	92.13 ± 3.69^{b}	101.84 ± 4.07^{bc}
	3 (48)	111.07 ± 4.44^{c}	82.76 ± 3.31^{a}	97.89 ± 3.92^{b}	87.67 ± 3.51^{a}	97.89 ± 3.92^{b}
	4 (72)	105.39 ± 4.22^{bc}	108.39 ± 4.34^{c}	107.92 ± 4.32^{bc}	137.85 ± 5.51^{e}	124.03 ± 4.96^{d}
Endoprotease activity at pH 9 (U g^{-1} f.m.)	2 (24)	59.49 ± 2.38^{d}	43.67 ± 1.75^{ab}	52.63 ± 2.11^{c}	46.00 ± 1.84^{b}	40.53 ± 1.62^{a}
	3 (48)	49.33 ± 1.97^{b}	46.67 ± 1.87^{bc}	44.74 ± 1.79^{b}	47.95 ± 1.92^{bc}	44.74 ± 1.79^{b}
	4 (72)	82.89 ± 3.32^{e}	88.16 ± 3.53^{e}	88.31 ± 3.53^{e}	103.08 ± 4.12^{f}	101.49 ± 4.06^{f}

Means followed by different letters are significantly different at $\alpha < 0.05$. Each value represents the mean of 3 measurements (±SE).

In the control conditions, similar observations were made in the following days of germination. Considering the changes in the protein content in cotyledons from elicited sprouts, it could be seen that elicitation stopped the mobilization of proteins (Figure 1a). Three days after induction with ions, the level of soluble protein was still over 20% higher than in cotyledons from control sprouts. According to DOMASH et al. (2008), this may be linked to the plant's stress response, including production of stress proteins e.g. protease inhibitors. Paradoxically, the level of non-protein nitrogen in some cases was significantly elevated by induction with the analyzed abiotic elicitors. For instance, 24 hours after treatment with 100 mmol dm^{-3} $CaCl_2$, 10 mmol dm^{-3} $MgCl_2$ and 100 mmol dm^{-3} $MgCl_2$, the level of peptide and free amino acids was higher by about 60.20%, 47.57% and 31.39%, respectively. The highest elevation of non-protein nitrogen was observed in cotyledons from 4-day-old sprouts induced with 10 mmol dm^{-3} $MgCl_2$ (475.7 mg g^{-1} f.m.) – Figure 1b. The elevation of the non-protein nitrogen fraction was probably due to the proteolysis of damaged and dysfunctional proteins (membrane protein–lipid complexes, the nuclear matrix and other structures).

In comparison to the control sprouts, multiple proteolytic systems were activated only in cotyledons from 4-day-old sprouts elicited with $MgCl_2$ solutions. A significant increase occurred in the activity of aminopeptidase and endoprotease (Table 2). Statistical analysis of the Pearson's correlations showed that the non-nitrogen content was positively correlated with the activity of acidic endoproteases and aminopeptidases in control sprouts (Table 3). These findings agree with the ones presented by MUNTZ et al. (2001) on protein mobilization during germination of leguminous seeds. The correlation coefficients indicated that samples from $MgCl_2$ solutions treatment contained the highest levels of non-protein nitrogen. It may be speculated that the above conditions caused extensive damage to protein structures, and also that other classes of proteases are involved in protein cleavage. Other authors

Table 3

Relationships between soluble protein and non-protein nitrogen content and proteolytic activity

Specification	Control	10 mmol dm^{-3} $CaCl_2$	100 mmol dm^{-3} $CaCl_2$	10 mmol dm^{-3} $MgCl_2$	100 mmol dm^{-3} $MgCl_2$
N-PN /AP	0.98	-0.08	0.34	0.91	0.93
N-PN / EP 5.5	0.57	0.33	0.75	0.99	0.83
N-PN / EP 7	0.43	0.36	0.79	0.99	0.81
N-PN / EP 9	0.08	0.16	0.55	0.95	0.84
SP / AP	-0.14	0.42	0.55	0.97	0.10
SP / EP 5.5	-0.97	0.01	0.08	0.88	-0.12
SP / EP 7	-1.00	-0.02	0.02	0.87	-0.17
SP / EP 9	-0.96	0.18	0.35	0.94	-0.10

N-PN – non-protein nitrogen, AP – aminopeptidase activity, EP 5.5 – endoprotease activity at pH 5.5, EP 7 – endoprotease activity at pH 7, EP 9 – endoprotease activity at pH 9.

reported a key role of protease in protein metabolism during abiotic stresses (Antão Malcata 2005, Palma et al. 2002).

Table 2 shows that the activity of proteases slightly decreased in the first days after induction. This effect was distinctly seen in cotyledons from 3-day-old sprouts, where activity of proteases was significantly decreased in comparison to control sprouts.

CONCLUSIONS

In the present research, it has been proven that elicitation with $CaCl_2$ and $MgCl_2$ solutions can be used to modify the rate of protein mobilization and may cause subsequent increase in the non-protein nitrogen fraction in cotyledons of germinated lentil.

1. Treatment with $MgCl_2$ solutions proved to be the most beneficial, leading to the highest yield of non-protein nitrogen in cotyledons from 6-day -old lentil sprouts.

2. Regardless of the type of elicitor, a decrease of the proteolytic activity was observed in cotyledons from 3- and 4-day-old lentil sprouts.

3. In sprouts elicited with $MgCl_2$ solutions, a significant correlation was found between the content of non-protein nitrogen content and activity of endo- and aminopeptidases.

The information provided by this study helps to design conditions for lentil sprouting which will result in production of food with an enhanced level of potentially bioaccessible non-protein nitrogen fraction (free amino acids and peptides).

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