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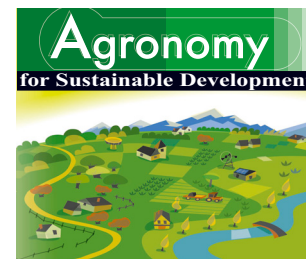
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Research article

Effect of plant species and temperature on amino acid release from plant material

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Abstract – Agricultural crop production is largely dependent on inputs of mineral fertilizers. Mineral fertilizer prices are expected to rise as the competition for fossil energy increases. In order to increase the sustainability of crop production, alternatives to commercial mineral fertilizers are needed. Organic residues and fresh biomass are potentially important sources of nitrogen (N) in crop production. A study was conducted to investigate the impact of temperature on the release rate and profile of amino acids from soybean seeds (*Glycine max* L.) and fresh young shoots of chicory (*Cichorium intybus*), alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) under anaerobic conditions. Plant material was incubated in water at either 15 °C or 35 °C, and the temporal release of total N, inorganic N, organic N and amino acids was measured during the 240 h incubation period. Amino acids and other organic N sources constituted significant proportions (20–84%) of the soluble N that was released. The contribution of organic N compounds to total N released decreased when the incubation temperature was raised from 15 °C to 35 °C, whereas the increase in temperature resulted in a higher total N release for only alfalfa and soybean. Amino acid profiles differed with plant material, incubation temperature and incubation length, with release patterns that only in a few cases could be described by first-order kinetics. Irrespective of the source, short-term liquid composting solutions will contain a low amino acid:inorganic N ratio with multiple amino acids present, which has to be taken into account when calculating the fertilizer effect. To conclude, this study reports, to our knowledge for the first time, the multiple amino acid release profiles from four different plant materials incubated under anaerobic conditions. The findings demonstrate that amino acids constitute significant proportions of the total N released and that the release patterns only for some amino acids followed first-order kinetics.

plant soaking / nitrogen release / amino acid profile / organic nitrogen

1. INTRODUCTION

Agricultural crop production is at present highly dependent on inputs of mineral N fertilizers to sustain a high production (Smil, 1999), but alternative N sources to mineral N fertilizers generated through the energy-demanding Haber-Bosch process are needed (Tilman et al., 2002). Organic residues and fresh biomass are potentially important sources of N in crop production, especially for resource-poor farmers who cannot afford agro-chemicals (IAASTD, 2009).

Nitrogen (N) supply is a major driving force for crop production in agroecosystems and the release of inorganic N from decomposing organic matter in soils is so well studied that mechanistic models can accurately predict the rate of N mineralization based on the organic material composition and the site characteristics (e.g. Müller et al., 2003). In order to successfully manage organic matter as a nutrient source, the pa-

rameters that determine the release and uptake of N by crops must be identified.

Recently, the uptake of dissolved amino acids by both forest trees and field crops has been studied, demonstrating that plants are able to take up and utilize amino acids from the root zone (Owen and Jones, 2001; Persson and Näsholm, 2002; Thornton and Robinson, 2005). In low-input agriculture, such as organic farming which excludes the use of commercial inorganic fertilizers, extracts of various plant sources with an adequate N content could serve as a supplementary N fertilizer. Such plant extracts may be prepared and applied locally by farmers. Protein constitutes 10–35% of plant dry matter and is made up of up to 20 different amino acids (Damodaran, 1996), with free amino acids being present in root tissue at concentrations of around 10 mM (Jones and Darrah, 1994). Thus, amino acids represent a large input of N in systems relying on the recycling of organic matter and degradation of organic N sources to sustain crop production.

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Table I. Characteristics, soaking ratio and total added N in the plant materials. FW is fresh weight, and DM is dry matter of the plant materials.

Plant material	Plant total N (g N 100 g DM ⁻¹)	Dry matter content (g DM 100 g FW ⁻¹)	Soaking ratio (g FW l ⁻¹)	Total added N (mg N l ⁻¹)
Alfalfa herbage	4.85	16.50	25	199.8
Chicory herbage	5.61	7.86	50	221.7
Red clover herbage	3.71	12.21	50	226.0
Soybean seeds	6.91	89.24	20	1233

Amino acids are released in the degradation of proteins following incorporation of organic residues in the soil, in a process described by Verma et al. (1975). However, under aerobic conditions the amino acids are expected to have a half-life of only a few hours (Jones, 1999). Amino acids have, nevertheless, been isolated in agricultural soils (e.g. Mengel, 1996) and many crops have the ability to take up amino acids directly (El-Naggar et al., 2009; Lipson and Näsholm, 2001). It has previously been shown that irrigation with plant extracts increases biomass production and the mineral and organic N contents of plants (Gramss et al., 2003; Hasegawa et al., 2002), and recently El-Naggar et al. (2008) demonstrated that the N sources in such solutions containing amino acids can be efficient N fertilizers under field conditions.

The composting of organic waste has been practiced by gardeners and farmers for centuries, but also the use of liquid composting, i.e. anaerobic soaking of plant materials, is an established practice in a number of environments; although this area is under-researched. Sporadic studies report, however, that Kenyan farmers use pruning of leguminous trees and other N-rich sources as soaking materials. In Egypt, producers have been experimenting with using Persian clover (*Trifolium resupinatum*). The use of liquid composting is also normal practice in regions of Cambodia, Thailand and Korea using a variety of residues (e.g. Briddlestone and Gray, 1987; Ezeroye, 2000; Onduru et al., 2002). However, knowledge is lacking regarding the compositions of the acting compounds in such solutions and how the solutions can be used strategically in crop management.

The objective of the current work was to study the release pattern over time of N compounds from four plant materials with different protein contents and under various temperatures. It was hypothesized that: (1) extracts of plant materials would release amino acids following first-order kinetics parallel to degradation curves; (2) the temperature would influence the rate of release significantly, and (3) amino acid profiles from various young herbage and seed materials would differ based on degradation patterns under aerobic conditions (Palm et al., 2001).

Abbreviations

Arginine, ARG; glutamate, GLU; aspartate, ASP; serine, SER; histidine, HIS; glycine, GLY; threonine, THR; arginine, ARG; alanine, ALA; tyrosine, TYS; cystine, CYS; valine, VAL; methionine, MET; tryptophan, TRP; phenylalanine,

PHE; isoleucine, ILE; leucine, LEU; lysine, LYS; ammonium, NH₄⁺; ammonia, NH₃; nitrate, NO₃⁻; nitrite: NO₂⁻.

2. MATERIALS AND METHODS

2.1. Plant material

In the summer season of 2004, fresh shoot material from chicory (*Cichorium intybus*), alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) was collected from standing herbage collections at the experimental farm of the Faculty of Life Sciences, University of Copenhagen, Denmark (55° 40' 19 N, 12° 17' 32 E). The legumes were unfertilized, but chicory received 100 kg ammonium sulfate at the start of the growing season. Within half an hour after sampling, the shoot material was chopped into 4–6-cm pieces; subsamples of the fresh materials were stored at -18 °C for later N analysis. Soybean seeds (*Glycine max* L.) were obtained from Central Soya Protein, Inc., Aarhus, Denmark, and ground (mesh size 1 mm; Retsch GmbH & Co., Haan, Germany). Subsamples of the materials were dried to constant weight (80 °C) and analyzed for total N and dry matter content (Tab. I).

2.2. Soaking experiment

The fresh plant materials were soaked in 2000 mL deionized water in acid-washed dark glass bottles with soaking ratios of 50 g FW L⁻¹, 50 g FW L⁻¹, 25 g FW L⁻¹ and 20 g FW L⁻¹ for chicory, red clover, alfalfa and soybean, respectively (see Tab. I) with the aim of adding a total of 220 mg N L⁻¹ as shoot material N and 1200 mg N L⁻¹ as seed storage proteins. The higher N concentration aimed at for the soybean seeds was necessary in order to (i) have comparable plant:water ratios and (ii) resemble practical conditions when used at the farm level. The bottles containing the soaked materials were incubated in growth chambers at 15 and 35 °C to approach a quadrupling in reaction rates and thereby enhance release and degradation. Four replicates for each plant and temperature treatment were used, giving a total of 32 bottles. The bottle openings were sealed with Parafilm® (American National Can, Chicago, Illinois, USA).

2.3. Sampling and chemical analysis

Samples of 20 mL were collected from each bottle at 24, 48, 120, 168 and 240 h after initiating the incubation. Prior to

sampling, the bottles were carefully shaken and samples were collected from the liquid phase. The samples were immediately centrifuged for 10 min at 1500 rpm at room temperature and the supernatant was poured off and filtered using cellulose acetate membrane filters (0.35- μm single-use syringe filters; Sartorius AG, Goettingen, Germany). The pH of the solutions was measured and the solutions were stored at $-18\text{ }^{\circ}\text{C}$ before analysis for total-N, NH_4^+ , NO_3^- and amino acids.

Total N of the soaked plant material was determined by in-line digestion followed by flow injection analysis (AutoAnalyzer 3, Bran + Luebbe, Seal Analytical GmbH, Norderstedt, Germany), where the N compounds are oxidized in-line into NO_3^- using alkaline persulfate/UV digestion after which the NO_3^- was reduced to NO_2^- over copperized cadmium. The reduced NO_3^- plus original NO_2^- was then determined by diazotization with sulfanilamide under acidic conditions to form a diazonium ion. The diazonium ion was coupled with N-1-naphthyl ethylenediamine dihydrochloride. In the filtered extracts, NH_4^+ was determined according to Switala (1993) and NO_3^- according to Sechtig (1992). All measurements were made spectrophotometrically (LACHAT Instruments, Milwaukee, M.I.). The total N content of the plant material was analyzed using an elemental analyzer (FlashEA 1112, ThermoQuest S.p.A., Milano, Italy).

2.4. Quantitative determination of amino acids

Liquid samples were diluted ten times with deionized water after which amino acids were analyzed according to the procedure of Hendersen et al. (2000). A HPLC 1100 (Agilent Technologies, Inc., Santa Clara, USA) was used with a Zorbax Eclipse AAA column (4.6 m \times 150 mm, 3.5 μm). The amino acids ASP, GLU, ASN, SER, GLN, HIS, GLY, THR, CIT, ARG, ALA, TYR, CY2, VAL, MET, NVA, TRP, PHE, ILE and LEU were derivatized using o-phthalaldehyde (OPA) and the amino acids LYS, HYP, SAR and PRO were derivatized using 9-fluorenylmethyl chloroformate (FMOC). The reaction mixture was buffered at a pH of 10.2 using 0.4 N borate buffer. The mobile phases were (a) 40 mM Na_2HPO_4 ; the pH was adjusted to 7.8 using 10 N NaOH, and (b) a mixture of acetonitrile, methylhydroxide and water (45:45:10, v/v/v). A G1312A binary pump at a flow rate of 2 mL min^{-1} delivered the degassed solvents. The ratios of the combined mobile phases were varied throughout the run time (26 min) with the percentage of (a) being 100% until 1.9 min, then 43% until 18.1, then 0% until the end of the analysis. The column effluent passed through a G1315A fluorescence detector (FLD). The resolved compounds were detected at 450 nm (Excitation = 340) and the signal was programed to change to 305 nm after 15 min from the run time to suit the FMOC derivatized amino acids. Quantitative determination of the amino acids was achieved by comparing peak surface areas with those obtained with pure amino acid standard solutions (Fulka production GmbH, Switzerland) in the range 0–250 pM.

2.5. Statistics and data analysis

The general linear model procedure of the SAS 8.2 statistical software (SAS Institute 2004) was used to carry out the statistical analysis. Means were compared by the Duncan test ($p < 0.05$) if the analysis of variance showed significant main effects or interactions. The trends for total N, inorganic N and amino acids released were simulated with the single first-order kinetic model:

$$N(t) = N_A \cdot (1 - e^{-kt}).$$

The model was described by DeNeve et al. (1996), where N is the amount of released N as a function of the incubation time (t), and N_A and k are the equation parameters, which were calculated using the SAS non-linear procedure (SAS Institute Inc., 2004). From here on, NH_4^+ and NO_3^- -N will be collectively referred to as inorganic N. Organic N was calculated as the difference between total N and inorganic N.

In order to elucidate whether the amino acid profiles were species-, soaking temperature- or soaking time-dependent, multi-variance analysis was conducted using the software program Unscrambler[®] (CAMO Process A/S, version 9.5, Oslo, Norway) for principal component analysis (PCA). PCA was performed on mean-centered and normalized variance amino acid data. PCA models were validated using cross-validation by the leave-one-out procedure and suspected outliers were identified on the basis of both their position in the score plots and on their leverage and residual values (Esbensen, 2001).

3. RESULTS AND DISCUSSION

3.1. Influence of soaking management on quantitative N release

The total N release pattern from alfalfa, chicory and soybean materials to the liquid phase was similar at both temperatures, while red clover showed a rapid increase in the released N until 24 h, followed by stable or even decreasing N levels (Tab. II). Both organic N and amino acid N were found to constitute significant proportions of the soluble N present in the solutions (Tab. II). Organic N generally dominated the N pools for plant material incubated at $15\text{ }^{\circ}\text{C}$, whereas for the plant material incubated at $35\text{ }^{\circ}\text{C}$ the organic N only dominated at the early samplings for red clover and alfalfa, and the first sampling for soybean. Amino acid N in the extracts of the four plant materials was present in all solutions from the start of the incubation period onwards and remained in varying proportions of the soluble N at both incubation temperatures.

The release pattern of organic N paralleled inorganic N in almost all the treatments (data not shown). The inorganic N fraction was mainly composed of NH_4^+ ($P < 0.05$). Nitrate was detected at low concentrations during the first 48 h of incubation, after which it disappeared from the solution (data not shown). Generally, both the release of inorganic N and total N followed exponential trends and were well described by a first-order kinetic model (R^2 varying from 0.84 to 0.96 for inorganic N and 0.83 to 0.95 for total N) with the exception

Table II. Release of total N (mg L^{-1}) from the incubated plant materials divided into N pools incubated at 15 and 35 °C for 24, 48, 120, 168 and 240 hours. Generally total N increases with time, resembling 1st order kinetic release. Temperature increases the N release for all plant materials but red clover. Organic N is the dominating N pool with free amino acids making significant contributions in all cases. NH_4^+ dominates the inorganic N pool.

Incubation temperature	15 °C					35 °C					
	Hours of incubation	24	48	120	168	240	24	48	120	168	240
Alfalfa	Total N	44 ± 10	30 ± 4.2	51 ± 5.8	59 ± 7.4	57 ± 11	38 ± 3.6	80 ± 13	130 ± 26	158 ± 38	204 ± 13
	Inorganic N	10 ± 1.8	10 ± 2.0	21 ± 2.9	22 ± 5.0	23 ± 5.0	15 ± 1.3	21 ± 1.7	67 ± 17	108 ± 25	109 ± 24
	NO ₃ -N	0.7 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.1
	NH ₄ -N	9.5 ± 1.9	9.9 ± 2.0	20 ± 2.9	22 ± 5.0	23 ± 5.0	14 ± 1.3	20 ± 1.7	67 ± 17	108 ± 25	109 ± 24
	Organic N	34 ± 8.5	20 ± 2.7	30 ± 5.6	36 ± 3.7	34 ± 7.6	23 ± 4.3	59 ± 11	63 ± 14	50 ± 14	95 ± 23
Red clover	Free amino acids	22 ± 4.3	14 ± 1.6	17 ± 2.7	21 ± 2.4	22 ± 5.6	13 ± 3.5	41 ± 6.4	34 ± 14	12 ± 1.4	52 ± 16
	Total N	41 ± 6.3	50 ± 6.5	36 ± 2.2	49 ± 2.3	42 ± 6.4	27 ± 1.7	31 ± 4.6	24 ± 2.0	19 ± 1.8	20 ± 2.4
	Inorganic N	17 ± 1.4	18 ± 1.8	14 ± 0.9	21 ± 2.4	20 ± 1.2	9.9 ± 0.4	8.9 ± 0.6	10 ± 1.1	12 ± 0.5	14 ± 1.4
	NO ₃ -N	1.3 ± 0.0	1.2 ± 0.1	0.0 ± 0.0	0.7 ± 0.4	0.0 ± 0.0	2.4 ± 0.2	1.3 ± 0.0	0.6 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
	NH ₄ -N	15 ± 1.4	16 ± 1.7	14 ± 0.9	20 ± 2.1	20 ± 1.2	7.5 ± 0.6	7.6 ± 0.6	9.6 ± 1.0	12 ± 0.5	14 ± 1.4
Soybean	Organic N	25 ± 6.5	32 ± 7.8	22 ± 1.6	28 ± 3.1	21 ± 7.0	17 ± 1.8	22 ± 4.4	13 ± 2.9	7.4 ± 1.4	6.7 ± 1.6
	Free amino acids	17 ± 4.8	18 ± 5.2	12 ± 0.5	16 ± 3.0	14 ± 5.3	10 ± 1.4	13 ± 3.2	8.3 ± 2.1	3.5 ± 1.3	1.5 ± 0.7
	Total N	32 ± 6.6	17 ± 0.2	26 ± 4.1	56 ± 4.3	36 ± 4.9	51 ± 5.4	53 ± 13.8	159 ± 20	369 ± 52	424 ± 35
	Inorganic N	5.3 ± 1.1	6.3 ± 0.3	9.5 ± 0.3	19 ± 1.0	21 ± 4.3	16 ± 0.6	27 ± 7.2	117 ± 9.1	274 ± 46	292 ± 18
	NO ₃ -N	1.9 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	1.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	0.2 ± 0.0
Chicory	NH ₄ -N	3.4 ± 1.1	4.9 ± 0.4	8.3 ± 0.3	18 ± 1.1	20 ± 4.2	14 ± 0.4	26 ± 7.3	117 ± 9.1	274 ± 46	292 ± 18
	Organic N	27 ± 6.8	10 ± 0.6	16 ± 4.0	37 ± 4.8	15 ± 1.7	35 ± 5.4	26 ± 7.0	42 ± 12	95 ± 19	132 ± 24
	Free amino acids	19 ± 3.8	6.8 ± 0.6	9.3 ± 2.4	29 ± 5.3	8.8 ± 2.0	23 ± 6.4	14 ± 4.2	5.1 ± 1.2	16 ± 2.6	13 ± 2.5
	Total N	2.0 ± 0.4	2.7 ± 0.8	1.6 ± 0.3	4.6 ± 0.6	6.4 ± 1.0	2.3 ± 0.7	2.4 ± 0.5	4.8 ± 0.7	7.6 ± 0.9	7.6 ± 1.6
	Inorganic N	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.2	2.8 ± 0.3	2.9 ± 0.3	1.3 ± 0.2	1.6 ± 0.3	2.6 ± 0.6	5.4 ± 0.7	6.2 ± 1.3
Chicory	NO ₃ -N	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	NH ₄ -N	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.2	2.8 ± 0.3	2.9 ± 0.3	1.1 ± 0.2	1.6 ± 0.3	2.5 ± 0.6	5.4 ± 0.7	6.1 ± 1.3
	Organic N	0.9 ± 0.3	1.4 ± 0.7	0.6 ± 0.1	1.8 ± 0.5	3.5 ± 0.8	1.0 ± 0.6	0.7 ± 0.2	2.2 ± 0.7	2.2 ± 0.3	1.5 ± 0.4
	Free amino acids	0.4 ± 0.2	0.9 ± 0.1	0.1 ± 0.1	0.7 ± 0.4	1.7 ± 0.4	0.6 ± 0.5	0.2 ± 0.1	1.3 ± 0.5	0.1 ± 0.0	0.1 ± 0.0

of red clover at 35 °C, where a decrease in total N was found after incubation for 48 h.

The current study shows that the release of inorganic N from alfalfa, chicory and soybean materials gradually increased from the first 24 h to 240 h (Tab. II). Despite no apparent similarities between the materials, these results agree with the findings of Hadas and Rosenberg (1992), which showed that the mineralization of guano in solution proceeded gradually, in contrast to its mineralization in soils, where 80% of the added N was recovered as inorganic N during the first three days of incubation. The decrease in the organic N pools for red clover without a similar increase in the inorganic N pools could indicate a loss of N via denitrification.

The increase in incubation temperature from 15 to 35 °C was expected to quadruple the release rate of N from the solid phase, which turned out to be the case for alfalfa ($P < 0.05$). For soybean, a tenfold increase in N release followed the increase in incubation temperature ($P < 0.05$). In contrast, the incubation temperature had no significant effect on the total N released for chicory and red clover (Tab. II).

The N extraction efficiency (Fig. 1) varied significantly ($P < 0.05$) among plant materials, although the total added N in fresh herbage material was similar (Tab. I). After 10 d of incubation, the highest N extraction efficiency for 15 °C incubation was recorded in alfalfa extracts with an average of $29 \pm 6.0\%$ of the added N, followed by 19 ± 3.2 , 3.0 ± 0.5 and $2.9 \pm 0.4\%$ of the added N for red clover, chicory and soybean, respectively. For plant materials incubated at 35 °C, the highest N extraction efficiency was detected in the alfalfa extracts with an almost complete extraction at $102 \pm 8.5\%$ of the added N, followed by 34 ± 2.9 , 9.0 ± 0.7 and $3.7 \pm 1.0\%$ of the added N for soybean, red clover and chicory, respectively.

Up to 30% of the total N was released within 50–100 h for the leguminous shoot material, which is consistent with a soil incubation study by Magid et al. (2004). Soybean seeds with a significantly higher N content had a lower N extraction efficiency than alfalfa ($P < 0.05$) (Fig. 1). This difference, and the differences between alfalfa, red clover and chicory incubated with similar N amounts, suggests that the structure and chemical composition of the incubated plant materials are important for its nutrient release under the current experimental conditions, which corresponds to studies on degradation of plant materials under aerobic conditions (e.g. Handayanto et al., 1997).

Aerobic systems are generally more microbiologically active than anaerobic systems. Hence the plant structure and chemical or biological degradability of plant compounds may be more important in anaerobic systems than in aerobic systems. In our study, the greater potential of alfalfa to release N and amino acids compared with the other plant materials may be due to (i) alfalfa plants having a higher proteolytic activity than the other crops (Jones et al., 1995a; Papadopoulos and McKersie, 1983), which enhances the protein autolysis, and consequently the release of amino acids and inorganic N, and/or (ii) a relatively high polyphenolic content in the tissues of red clover and chicory, which reduces the efficiency of proteolysis enzymes (Jones et al., 1995a, b, c). Alfalfa leaves and

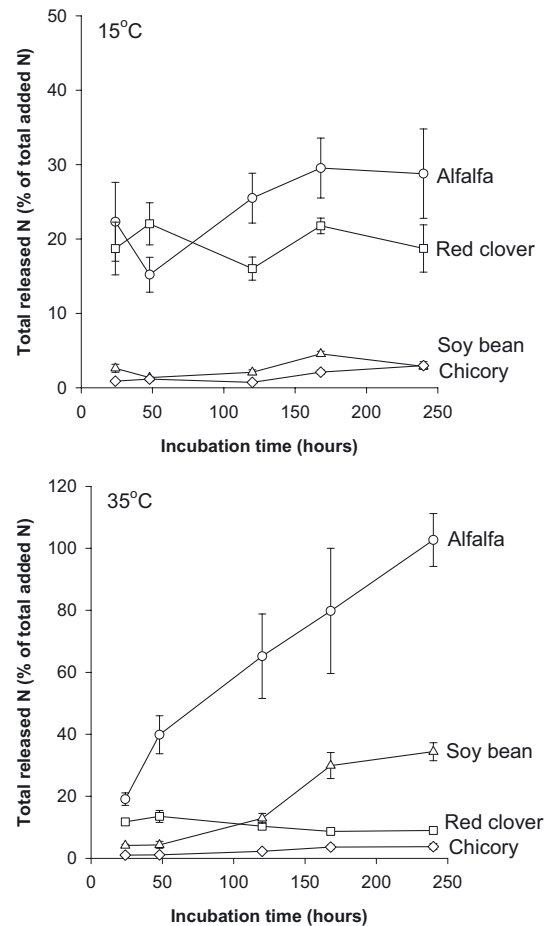


Figure 1. Total N release over time as a proportion (%) of the initial amount of incubated N in alfalfa (○), chicory (◇), red clover (□) and soybean (Δ) applied as fresh materials at two incubation temperatures, 35 °C (A) and 15 °C (B). Alfalfa has the highest extraction efficiency at both incubation temperatures, reaching almost 100% after 240 h of incubation at 35 °C. Soybean has a tenfold increase in extraction efficiency when increasing the temperature from 15 °C to 35 °C. Red clover and chicory are unaffected by the temperature increase.

stems have little, if any, polyphenol oxidase activity (Sullivan and Thoma, 2004).

Chemical processes have been found to dominate the N release in degradation studies of winter season cover crops (rye and vetch: Ruffo and Bollero, 2003) and with disproportionately high release rates for *Medicago lupulina* even at low temperatures (Magid et al., 2004). This also tallies with the statement that several proteins undergo dissociation and denaturation under low temperatures (Damodaran, 1996). However, the lack of temperature effect on the release rate for red clover and chicory cannot be understood based on the currently available data.

Table III. Peak concentration and time for peak for the 16 detected amino acids from the plant materials incubated at 15 °C. "I" when increasing, and "D" when decreasing with time indicate the general trends in amino acid presence in the extracts. Amino acids not detected are indicated by "n.d.". In extracts from soybean seeds all 16 amino acids were found, whereas the chicory extracts only had five detectable amino acids all in low concentrations. The same 14 amino acids were detected in alfalfa and red clover extracts. ARG and GLU were the most abundant amino acids in the legume extracts.

Amino acid	Alfalfa		Red clover		Soybean		Chicory		
	Peak conc. mg L ⁻¹	Time hours	Peak conc. mg L ⁻¹	Time hours	Peak conc. mg L ⁻¹	Time hours	Peak conc. mg L ⁻¹	Time hours	
ASP	18 ± 6.7	24	D	12 ± 3.1	24	D	6.1 ± 1.1	168	n.d.
GLU	18 ± 1.7	240		14 ± 3.3	48		27 ± 4.8	168	n.d.
SER	6.6 ± 1.8	24	D	3.4 ± 1.3	24	D	1.1 ± 0.5	24	n.d.
HIS	11 ± 2.1	240	I	7.3 ± 1.4	168		16 ± 2.8	168	0.5 ± 0.5 240
GLY	2.6 ± 0.6	24		1.7 ± 0.6	24		3.9 ± 1.3	168	n.d.
THR	1.4 ± 0.5	240	I	0.3 ± 0.3	24	D	7.2 ± 2.2	24	n.d.
ARG	28 ± 7.5	240		25 ± 7.9	48		32 ± 5.5	168	1.9 ± 1.0 240 I
ALA	13 ± 2.4	24	D	9.3 ± 3.7	48	D	13 ± 3.8	168	2.6 ± 0.5 240
TYR	5.0 ± 1.7	240		1.3 ± 1.3	48		15 ± 5.3	168	n.d.
CYS	10 ± 2.8	240		7.7 ± 1.1	168		8.0 ± 2.5	168	2.0 ± 0.2 240
VAL	n.d.			n.d.	–		2.5 ± 0.8	24	n.d.
MET	3.3 ± 0.9	240		1.5 ± 0.6	24		3.5 ± 0.7	168	n.d.
TRP	n.d.			n.d.	–		2.4 ± 2.3	168	n.d.
ILE	3.2 ± 0.4	120		2.3 ± 0.8	48		4.8 ± 1.5	24	n.d.
PHE	8.6 ± 1.3	240		4.8 ± 1.5	24		5.1 ± 1.0	168	n.d.
LEU	11 ± 2.2	240	I	5.0 ± 1.2	168		7.7 ± 1.5	24	n.d.

3.2. Influence of soaking management on released amino acid profiles

A total of 16 amino acids (Tabs. III and IV) out of 20 possible were identified in the extracts with amino acid profiles and with concentrations varying significantly ($P < 0.05$) with source, temperature and soaking time. The seven most abundant of the amino acids were ASP, GLU, SER, HIS, GLY, ARG and ALA.

Soybean seed material soaked at 15 °C gave an amino acid profile dominated by ARG and GLU. All amino acids were present at the 24 and 168 h samplings. Generally, most amino acids showed the highest concentrations at the 168 h sampling. At 35 °C ARG and ALA dominated after 24 h; thereafter, the concentration of TYR increased markedly, becoming the main amino acid present in the soaking solution. All analyzed amino acids were found in the soaking solution, with GLU, GLY, THR, ARG and ALA decreasing and TYR increasing with time of soaking. The PCA showed clear separation between samples incubated at 15 °C and 35 °C (Fig. 2A), with TYR, VAL and TRP being identifiers of samples from 35 °C, and GLU, ARG, ASP, THR and HIS from 15 °C (Fig. 2B). Also, the time of soaking affects the amino acid profiles. This is shown in Figure 3 for soybean soaked at 35 °C where the early samples are concentrated below the dotted line in the score plot (Fig. 3A), while the later samplings can be found in the top left. The early samplings are identified by amino acids such as ARG, GLU and ALA, whereas TYR, TRP and VAL are specific to the later samplings (Fig. 3B).

The amino acids released from alfalfa material soaked at 15 °C were dominated by ARG and GLU, whereas VAL and TRP were not detected. Most amino acids were present at fairly constant concentrations at all sampling times, with the exception of HIS, THR and LEU that showed increasing and ALA, ASP and SER decreasing concentrations during the sampling period; the latter two almost disappearing from solution after 240 h. Amino acids from alfalfa soaked at 35 °C were generally dominated by ARG, GLU and HIS, with TYR, ILE, PHE and LEU increasing markedly with duration of soaking. After 48 h of soaking, all 16 amino acids were present in solution, although at this sampling VAL, MET, TRP, GLY and THR were found at low concentrations. As with alfalfa soaked at 15 °C, ASP and SER had high initial concentrations, which decreased over time. The PCA (data not shown) showed separation between the samples incubated at 15 °C and 35 °C, although not as clear as for soybean. Likewise, the PCAs showed that samples differed in amino acid profiles in relation to soaking time. The early samplings for both 15 °C and 35 °C were identified by ALA, SER and ASP, whereas the later samplings were identified at 15 °C by THR and at 35 °C by, among others, PHE, HIS and TYR.

Soaking of chicory at both 15 °C and 35 °C gave rise to only four amino acids (HIS, ARG, ALA and CYS) and all four were at small concentrations. For 15 °C the highest amounts were present at the final sampling after 240 h, whereas for 35 °C the highest amounts were seen after 120 h with no amino acids left after 240 h.

The profile of amino acids from red clover material soaked at 15 °C was dominated by ARG and GLU, with HIS, ALA

Table IV. Peak concentration and time for peak for the 16 detected amino acids from the plant materials incubated at 35 °C. “I” when increasing, and “D” when decreasing with time indicate the general trends in amino acid presence in the extracts. Amino acids not detected are indicated by “n.d.”. In extracts from soybean and alfalfa seeds all 16 amino acids were found, whereas red clover extract had 13, and chicory extracts had five detectable amino acids. ARG were among the most abundant amino acids for all extracts.

Amino acid	Alfalfa			Red clover			Soybean			Chicory	
	Peak conc. mg L ⁻¹	Time hours		Peak conc. mg L ⁻¹	Time hours		Peak conc. mg L ⁻¹	Time hours		Peak conc. mg L ⁻¹	Time hours
ASP	16 ± 1.9	48	D	3.1 ± 0.5	24	D	2.3 ± 0.5	24		n.d.	
GLU	52 ± 11	240		8.5 ± 1.7	48	D	10 ± 4.8	24	D	n.d.	
SER	13 ± 2.6	48	D	1.5 ± 0.3	24	D	1.5 ± 1.1	168		n.d.	
HIS	36 ± 11	240		6.8 ± 1.9	48	D	2.8 ± 1.0	24		0.8 ± 0.4	120
GLY	6.4 ± 0.9	48	D	0.7 ± 0.4	24	D	5.4 ± 1.0	24	D	n.d.	
THR	3.9 ± 1.8	240		n.d.			6.2 ± 2.1	24	D	n.d.	
ARG	42 ± 22	240		17 ± 4.0	48	D	28 ± 8.5	24	D	1.8 ± 0.8	120
ALA	14 ± 2.4	48		6.6 ± 2.2	48	D	20 ± 5.0	24	D	1.6 ± 1.3	24
TYR	29 ± 6.0	240	I	5.8 ± 2.0	168	I	68 ± 20	168	I	n.d.	
CYS	27 ± 5.9	240		5.9 ± 1.4	48		11 ± 3.1	24		1.5 ± 0.2	120
VAL	3.3 ± 0.4	48		n.d.			19 ± 4.5	168		n.d.	
MET	4.8 ± 1.9	120		0.5 ± 0.5	48		5.5 ± 3.4	48		n.d.	
TRP	4.1 ± 1.6	48	D	n.d.			24 ± 4.4	168		n.d.	
ILE	22 ± 5.9	240	I	2.0 ± 0.9	48		5.2 ± 1.7	24		n.d.	
PHE	42 ± 7.9	240	I	3.2 ± 1.2	48		4.0 ± 1.4	48		n.d.	
LEU	40 ± 9.9	240	I	6.1 ± 2.0	48		7.6 ± 1.6	48		n.d.	

and CYS also found in fair amounts. ASP and SER had high initial concentrations which decreased over time, whereas none or only insignificant amounts of THR, VAL and TRP could be detected at any time. The 35 °C treatment gave a profile also dominated by ARG and GLU, although at this temperature the concentrations of these two amino acids decreased markedly at the 168 and 240 h samplings. ASP, GLU, SER, GLY, HIS, ALA and ARG had relatively high concentrations after 24 and 48 h, but decreased at the later samplings. In contrast, TYR increased in concentration with duration of soaking. The PCA (data not shown) showed separation between the samples incubated at 15 °C and 35 °C, although there was more overlap between temperature groups. PCAs showed that samples taken after 24 and 48 h clearly differed more in amino acid profiles than the later samplings. The early samplings for both 15 °C and 35 °C were identified by, among others, SER and ASP, whereas the later samplings were identified at 15 °C by HIS and at 35 °C by, among others, MET and TYR.

For all four plant materials, the soaking temperature and duration had a large effect on amino acid profiles. No amino acid was generally representative for either 15 °C or 35 °C, or for early and later sampling times. Chicory was clearly different from the other three species as only four amino acids were found, all at low concentrations. Alfalfa and red clover had similar amino acid profiles when soaked at 15 °C, and were only different from the soybean amino acid profile in the timing of peak concentrations (Tab. III). However, at 35 °C a larger degree of differentiation was found between alfalfa, red clover and soybean. THR, VAL and TRP were found in alfalfa and soybean solutions, but not in red clover solutions. The time of peak concentrations of the dominating amino acids showed

marked differences between red clover and alfalfa, with red clover peaking at the beginning of the incubation and alfalfa at the end.

ASP and SER had similar temporal trends of appearance across incubation temperatures and plant materials (Fig. 4A). ARG, GLU, HIS and CYS likewise formed a group in terms of pattern of appearance (Fig. 4B), which was also the case for ILE, PHE and LEU (Fig. 4C).

The profile of amino acids in the extracts shows the advantage of using alfalfa materials compared with the other tested materials. The high concentration of amino acids in alfalfa extracts supports our previous discussion on the enhancement of N release from the plant material caused by higher proteolytic activity in alfalfa. Unlike the NH₄⁺-N and the total N released, amino acid N concentrations did not show clear temporal trends. We therefore conclude that the amino acid N concentration, in contrast to our hypothesis, was not well described by first-order kinetics. The multivariate data analysis demonstrates that amino acids differed in their degradation rate. This was affected by time and temperature (Figs. 2, 3) as, i.e., ALA, ASP and SER showed decreasing concentrations during the sampling period (Tabs. III, IV). These findings are in agreement with Sato et al. (2004), who under highly different experimental conditions found that ALA (and its derivatives LEU, PHE, SER and ASP) was degraded under high temperatures with different degradation rates. Sato et al. (2004) suggests enzymatic oxidative deamination and decarboxylation as the driving processes, but in our study other processes such as retention in the microbial biomass or in the solid phase may have been responsible. The degradation rate decreased in the following order: ASP, SER, PHE, LEU and ALA. This is also

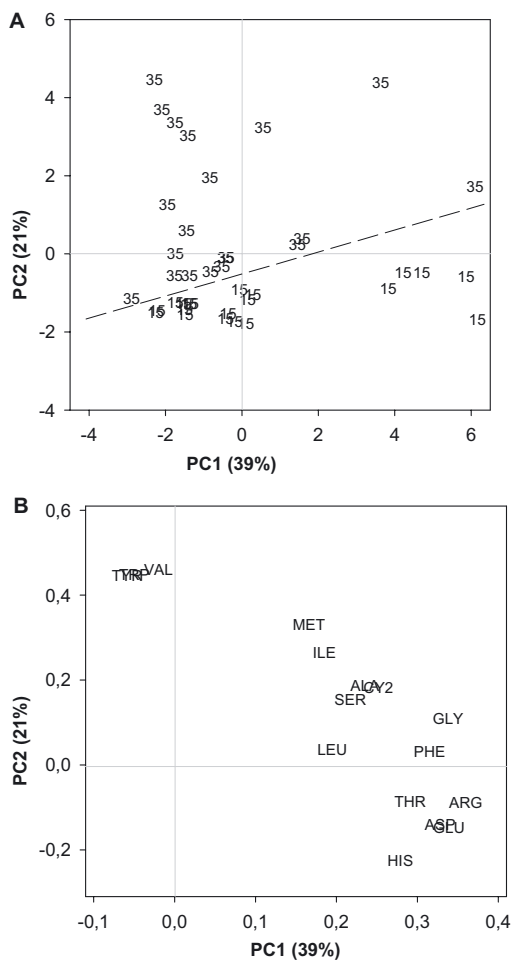


Figure 2. Amino acids from all soybean grain samples analyzed by principal component analysis (PCA) to exemplify the method of identifying amino acids specific to each of the two soaking temperatures. Score plot (A) shows the differentiation between 15 and 35 °C, and loading plot (B) shows that TYR, TRP and VAL to the upper left are identifiers for 35 °C and THR, ARG, ASP, GLU and HIS at the lower right are identifiers for 15 °C.

clear from Figure 2, where ALA and its derivatives dominate the soybean solution at 15 °C, while the phenolic amino acids dominate the 35 °C incubated plant soakings.

The findings in our study showed that the three legume materials, as expected from the N content, gave the highest yields of released N. Due to the high cost of soybean seeds we anticipate that red clover and especially alfalfa will be the most attractive materials to be used as supplementary fertilizers in organic farming. Recent research has shown special preferences among plant species for amino acid uptake (Weigelt et al., 2005; Persson and Näsholm, 2001), which implies that further research is needed to determine the plant material with the most suitable amino acid profiles to fertilize a given plant species.

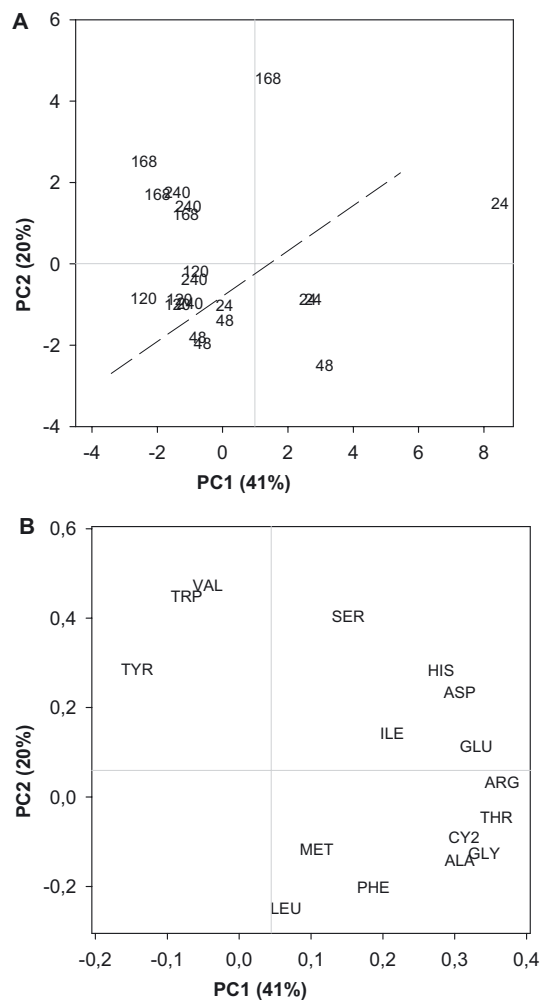


Figure 3. Amino acids from soybean grain samples soaked at 35 °C analyzed by principal component analysis (PCA) to exemplify the method of identifying amino acids specific to the time of incubation. Score plot (A) shows the differentiation between 24 and 48 h, and 120, 168 and 240 h of incubation, below and above the dotted line, respectively. The loading plot (B) shows that TYR, TRP and VAL to the upper left are identifiers for longer incubation times (120, 168 and 240 h) and, e.g., ARG, GLU and ALA to the lower right are identifiers for the shorter incubation times (24 and 48 h).

4. CONCLUSION

The current study reports, to our knowledge for the first time, the multiple amino acid release profiles from four different plant materials incubated under anaerobic conditions. The study demonstrates that amino acids constitute significant proportions of the total N released and that the release patterns only for some amino acids followed first-order kinetics. Temperature influenced the rate of release significantly for alfalfa and soybean but not for red clover and chicory. A total of 16 amino acids were identified in the plant extracts, but amino acid profiles from shoot material and seed materials differed ($P < 0.05$) with incubation time and temperature. ARG, GLU and ALA dominated the first flush of releases, whereas TYR,

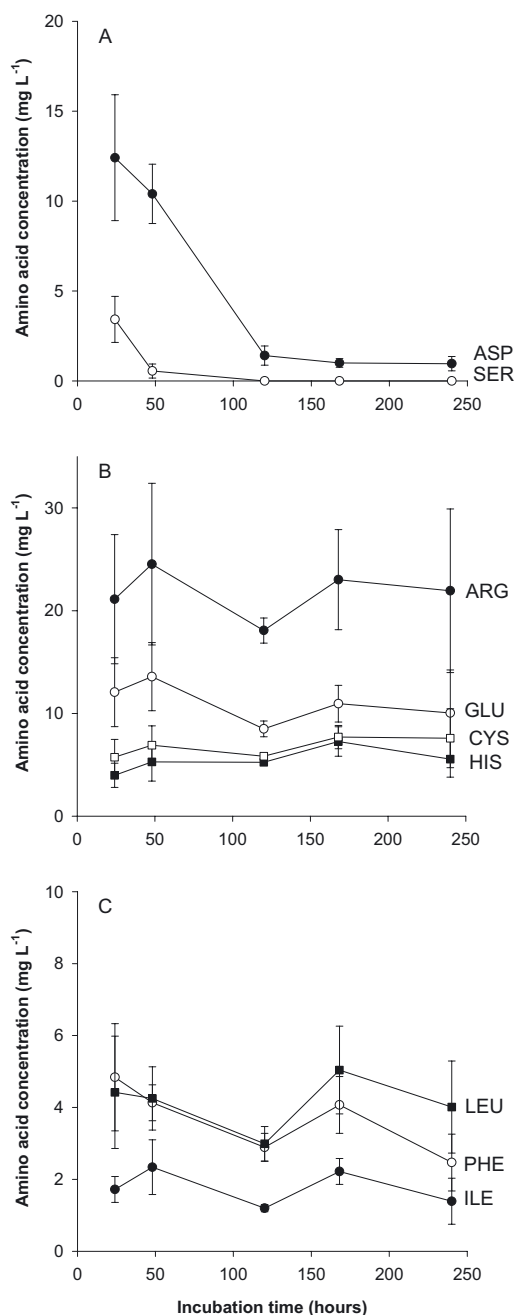


Figure 4. Temporal development in ASP and SER (A), ARG, GLU, HIS and CYS (B), and ILE, PHE and LEU (C) presence in the plant soakings. Exemplified with data from red clover at 15 °C showing that the three groups of amino acids have the same temporal appearance in the plant soaking.

TRP and VAL appeared later. Knowledge of the amino acid release profiles should be included in future studies where rapid N releases under anaerobic conditions are being considered.

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