ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XLIII, nr 1
1974

Soluble nitrogen compounds in tillering nodes and roots of *Dactylis glomerata*

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(Received: June 13, 1973.)

Abstract

A characteristic is given of the low molecular weight fraction of nitrogen compounds in tillering nodes and roots of the grass *Dactylis glomerata* in spring and in autumn. It was found that in vegetative storage organs of *Dactylis glomerata* glutamic acid with glutamine, aspartic acid with asparagine, alanine and arginine play important roles in the storage of nitrogen. From the free amino acids pool of the tillering nodes and roots ten amino acids in crystalline form were isolated and identified.

INTRODUCTION

Perennial green plants store nitrogen compounds in specially adapted vegetative storage organs — corms, tubers, bulbs etc. In these organs nitrogen is stored primarily in the form of low molecular weight nitrogen compounds, such as protein and nonprotein amino acids, amides and ureides (M c K e e, 1962). In the spring these compounds are carried to sites where they are needed.

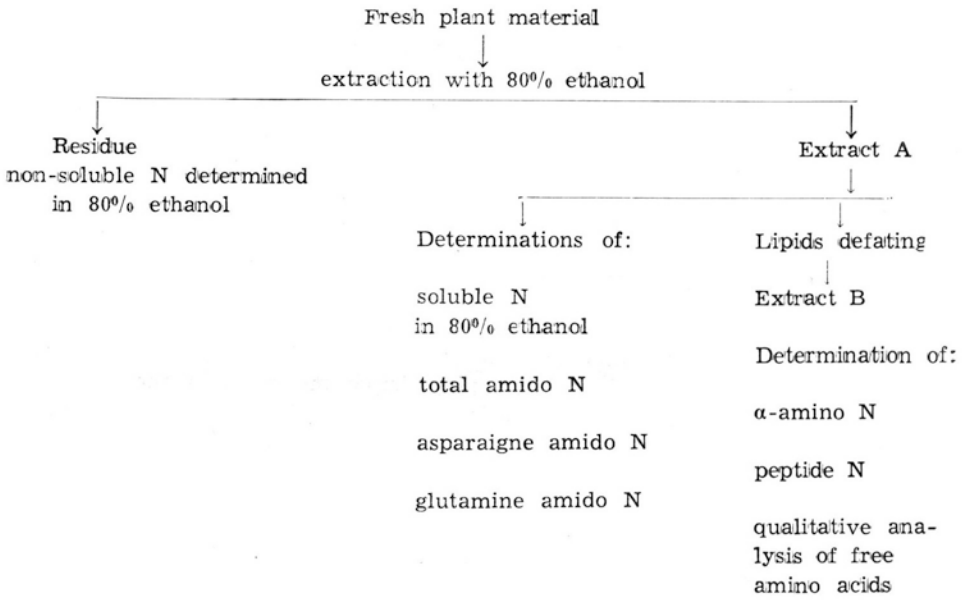
In the literature concerning nitrogen metabolism of grasses, data are lacking on the form of nitrogen storage in vegetative organs of perennial species. The present paper reports on investigations of the forms of soluble nitrogen in the tillering nodes and roots of one of the representatives of perennial grasses — *Dactylis glomerata*.

MATERIAL AND METHODS

Plant material

As object of study served plants growing in field conditions. The tillering nodes were analysed together with roots in spring and in autumn in three successive vegetation seasons (second, third and fourth year of development of the plants). Samples were collected in the years 1966—1968.

Scheme of analytical procedure



Analytical methods

Extraction of soluble nitrogen compounds from plant material and defating were performed as described by Przybylska and Rymowicz (1965).

Total nitrogen was determined in dry plant material by Kieldahl's method in Perrin's modification (1953).

Non-soluble nitrogen in 80 per cent ethanol was determined in the dry residue after extraction by Kjeldahl's micromethod (Bailey, 1962).

Soluble nitrogen in 80% ethanol determined as follows: a given volume of extract A was mixed with a corresponding volume of selenium mixture; after evaporation of ethanol the sample was

mineralized in an aluminium block for 6 h. Ammonia was distilled off in Parnas apparatus. Total nitrogen in the sample was distilled in the course of 4 min.

α-Amino nitrogen was determined in extracts B by Pope-Steven's method in the modification of Albanese and Irby (1944).

Peptide nitrogen was calculated as the difference between α-amino nitrogen content after drastic hydrolysis of the extract (hydrolysis conditions: 6 N HCl, 24 h, 100°C, under reflux) and α-amine nitrogen of the nonhydrolysed extract.

Total amido nitrogen. A known volume of extract A was hydrolysed with 1 N sulphuric acid for 3 h on a boiling water bath under reflux. The ammonia released during hydrolysis was determined by the diffusion micromethod of Conway (1947).

Glutamine amido nitrogen. A known volume of extract A was hydrolysed with borax-phosphate buffer pH 6.5 for 2 h under reflux on a boiling water bath (Reifer and Tarnowska, 1952). The ammonia released during hydrolysis was determined as described above. The amido nitrogen of glutamine was calculated as the difference between the amount of ammonia nitrogen liberated during hydrolysis with buffer of pH 6.5 and the ammonia nitrogen content in the nonhydrolysed extract.

The amido nitrogen of asparagine was calculated as the difference between total content of amido nitrogen and the amount of ammonia nitrogen content of liberated during hydrolysis of the extract in buffer of pH 6.5.

Ammonia nitrogen was determined in extracts A by the diffusion micromethod of Conway (1947).

Two-dimensional paper chromatography was applied in the qualitative analysis of the amino acid composition. This technique has been described by Przybylska (1964) and Przybylska and Rymowicz (1965).

Circular paper chromatograms were run in three solvent systems: 1) n-butanol-acetic acid-water 4:1:5 v/v, upper layer; 2) n-butanol-methylethylketone-ammonia-water 5:3:1:1 v/v; 3) phenol-ethanol-water 150:40:10 v/v.

Paper electrophoresis was carried out in acetate-pyridine buffer pH 4.5 (acetic acid-pyridine-water, 25:15:960 v/v) (Wagner 1958) on Whatman no. 3 paper for 6 h at 10 V/cm.

The chromatograms and electropherograms were developed in 0.4 per cent ninhydrin solution in acetone with 0.2 per cent cobaltous chloride added. For identification of some of the amino acids characteristic colour tests were applied. Arginine was identified by the colour reaction with Sakaguchi's reagent (Smith, 1960), β-alanine and

γ-aminobutyric acid by the reaction with Larsen and Kjaer's reagent (1960), tryptophane on the basis of Ehrlich's reaction (Smith, 1960), histidine by the reaction with Pauly Ehrlichs reagent (Block, Durrum, Zweig, 1958), and phenylalanine by means of the ninhydrin reagent after Pasieka and Morgan (Greenstein and Winitz, 1962).

Isolation of the amino acids. Five kilograms of tillering nodes and roots were homogenised with a small amount of 96 per cent ethanol and extracted five times with 6 l. portions of cold 80 per cent ethanol and once with a 6 l. portion of hot ethanol. The combined extracts were evaporated in a rotary evaporator at 40°C. The obtained dark syrup (234 g) was dissolved in 1 l. of water and after filtering off the insoluble part, the solution applied on a column of strongly acid ion-exchange resin (Dowex 50-X8, 200-400 mesh) in H^+ form. The total was applied on four columns (each 4,5 × 45 cm). After washing the columns with water, the amino acids were eluted with 1 N ammonia solution and 25-ml fractions were collected. The fractions showing a positive reaction with ninhydrin were combined and evaporated on a rotary evaporator at 40°C. The obtained dark syrup (102 g) was dissolved in 800 ml of water; for purifying the free amino acid fraction the solution obtained was once more subjected to ion-exhange chromatography on Dowex 50-X4 (H^+) as described above. The eluate containing the amino acids was evaporated in a rotary evaporator at 40°C to a thick syrup (94 g).

Isolation of aspartic and glutamic acids. The dark syrup was dissolved in 500 ml of water and applied on a column of strongly basic ion-exchange resin (Dowex 1-X8; 200-400 mesh) in acetate from. The total was applied on two colums (each 1.8 × 65 cm). Each column was washed with 1500 ml of CO_2-free water, the effluent and the washings were collected in 25-ml fractions. As shown by paper electrophoresis, these fractions contained only neutral and basic amino acids.

Acidic amino acids were eluted with an increasing concentration of acetic acid, the eluates were collected in 20-ml fractions (0.1 M, 0.25 M, 0.3 M, 0.5 M and 1 M, 600 ml each).

Fractions 38—46 contained glutamic acid, fractions 69—75 aspartic acid and small quantities of γ-glutamyl-glutamine *. Fractions containing glutamic acid were combined and evaporated to dryness under reduced pressure. After two recrystallizations from water 4.6 g of glutamic acid crystals were obtained.

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* Isolation of γ-glutamyl-glutamine has been earlier described (Dąbrowska, 1971 b).

Franctions containing aspartic acid with a small amount of γ-glutamyl-glutamine were combined and evaporated to dryness as described above. Aspartic acid was crystallized from water; after two recrystallization 2.5 g of crystal were obtained.

Isolation of tryptophane, γ-aminobutyric acid, histidine, ornithine, lysine and arginine. The fractions constaining neutral and basic amino acids were evaporated under reduced pressure at 40°C to a thick syrup (64.7 g). After dissolution of the syrup in 300 ml of water, the solution was applied on a column of strongly acid ion-exchange resin (Dowex 50-X4, 20 - 400 mesh) in ammonia form. The total was applied on two columns (2.8 × 50 cm each). Each column was washed with 1300 ml of water; the effluent and water washings were collected in 20-ml fractions.

As shown by paper electrophoresis the fractions 3 - 35 contained neutral amino acids. Basic amino acids were eluted with 800-ml portions of aqueous ammonia solution of the following concentrations: 0.05 N, 0.1 N, 0.25 N and 0.5 N. The eluates were collected in 20-ml fractions. Tryptophane, γ-aminobutyric acid and histidine were eluted with a 0.05 N solution (tryptophane: fractions 3 - 14; γ-aminobutyric acid: fractions 18 - 24; histidine: fractions 30 - 35), ornithine was eluted with 0.1 N solution (fractions 48 - 53), lysine with 0.25 N solution (fractions 83 - 90), arginine with 0.5 N solution (fractions 123 - 135).

The fractions containing the same amino acid were combined and evaporated to dryness on a rotary evaporator at 40°C. After repeated recrystallizations from water all the above mentioned amino acids were obtained in crystalline form. Lysine and ornithine were obtained in the form of hydrochlorides. The following amounts of crystalline amino acids were obtained: tryptophane 0.45 g, γ-aminobutyric acid 0.38 g, histidine 0.98 g, arginine 3.02 g, ornithine 0.15 g and lysine 0.52 g.

Isolation of asparagine and phenylalanine. The fraction of neutral amino acids was evaporated to a thick syrup (40 g). This syrup was dissolved in a small volume of water and left to stand for 24 h at 4°C. In these conditions asparagine crystallized. After filtration the asparagine crystals were subjected to twofold recrystallization from water and 4 g of chromatographically pure asparagine were obtained.

The solution of neutral amino acids (500 ml) after removal of about 80 per cent of asparagine by way of crystallization was applied on two columns of strongly acid ion-exchange resin (Dowex 50-X4, 200-400 mesh; each 30 × 50 cm prepared according to Lewis, 1966). Each column was washed with 1500 ml of citrate buffer pH 2.8. The effluent and eluate was collected in 25-ml fractions. As shown by two-di-

mensional paper chromatography fractions 10 - 45 contained asparagine, glutamine, analine, serine, β-alanine, proline, and theronine; fraction 47 - 53 contained phenylalanine; fractions 54 - 59 phenylalanine, methionine and/or valine, isoleucine and leucine.

Fractions containing only phenylalanine were combined and desalted on Dowex 50-X4 (200 - 400 mesh, 4.5 × 50 cm) in H^+ form. Phenylalanine was eluted with 0.5 N ammonia solution. The eluate was evaporated to dryness, ammonia was removed by repeated evaporation with water. After twofold recrystallization from 50 per cent ethanol 350 mg of phenylalanine crystals were obtained.

The IR absorption spectra determined in KBr discs on Unicam Sp. 200 spectrophotometer. Melting points were determined in sealed capillary tubes, heated 2°C per minute.

RESULTS AND DISCUSSION

1. Changes in the level of various nitrogen forms in tillering nodes and roots during spring and autumn

Data concerning the content of various nitrogen forms in tillering nodes with roots in the period of late autumn and early spring, collected in the years 1966 - 1968 are shown in diagram 1. It results from these data that the level of the particular nitrogen forms shows rather wide differences in the organs examined in depending on the season. The total nitrogen level is higher in autumn than in spring, this resulting from a higher contribution of nitrogen of non-soluble compounds (Diagram 1A). The differences in the contribution of particular of nitrogen soluble forms to total soluble nitrogen in dependence on the season are also noteworthy. As indicated in diagram 1 B in the spring period, tillering nodes with roots exhibit, a high contribution of α-amine nitrogen and a much larger than in autumn contribution of peptide nitrogen. In autumn a greater contribution of amido nitrogen is observed than in spring. This increase in amido nitrogen in the tillering nodes and roots in autumn is due primarily to the amido nitrogen of asparagine (Diagram 1C).

If we compare these results here obtained against the background of data for other organs (Dąbrowska 1971 a), it appears that tillering nodes and roots contribute particularly large amounts of soluble nitrogen compounds to the total nitrogen pool. In autumn the contribution of soluble nitrogen from these organs to total nitrogen content varies from 36 to 48 per cent depending on the year of harvest. This leads to the conclusion that in the vegetative storage organs of *Dactylis glomerata* nitrogen is stored primarily in the form of low

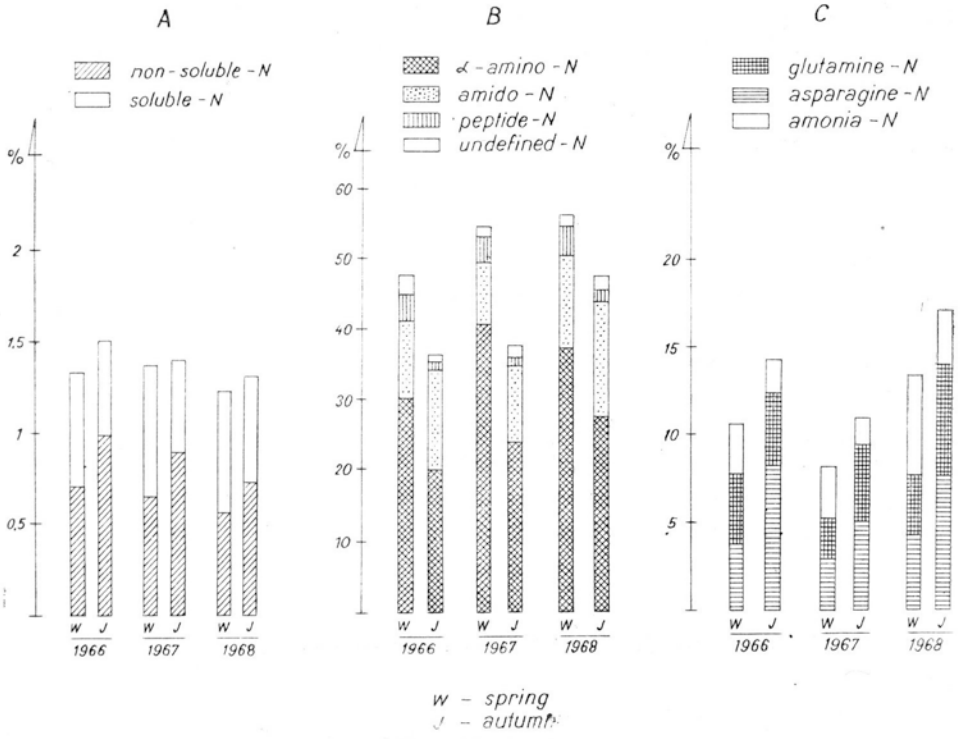

Diagram 1. Contents of different forms of nitrogen in tillering nodes with roots of *D. glomerata*. *A* — total nitronge, *B* — soluble nitrogen, *C* — total amido nitrogen. (Legend see p. 57)

molecular weight compounds. The results of analyses for the content of various soluble nitrogen forms indicate that nitrogen is mainly stored in the form α-amine and amido nitrogen. Peptide nitrogen does not play any major role in nitrogen storage in the examined species. This feature is typical for vegetative storage organs of many plant species (McKee, 1962; Mothes and Engelbrecht, 1952; Oland, 1959; Reuter, 1957; Steward, Wetmore, Thompson and Nitsch, 1954).

The decrease in the non-soluble nitrogen level observed in spring in the tillering nodes and roots with simultaneous increase in soluble nitrogen suggest that a certain part of non-soluble nitrogen may be a form of nitrogen storage which appears to be "mobilized" in the form of soluble compounds before vegetation starts.

2. Free amino acids in tillering nodes and roots in spring and autumn

Chromatographic analysis of free amino acids in tillering nodes and roots demonstrated that both in autumn and in spring the dominating amino acids were glutamic acid with glutamine, aspartic acid, alanine

and arginine. The contribution of serine to the pool of free amino acids was smaller. Lysine, histidine, proline methionine and/or valine, phenylalanine, leucine, threonine and tryptophane occurred in low or trace quantities. In the tillering nodes and roots collected in autumn asparagine also was found in large amounts.

Analyses performed separately for the tillering nodes and the roots demonstrated that the high arginine concentration in tillering nodes together with roots is the result of accumulation of large quantities of this amino acid in the nodes. Characteristic for tillering nodes was also a rather high concentration of free lysine.

These data suggest that among the free amino acids an important role in nitrogen storage in vegetative organs of *D. glomerata* is played by glutamic acid with glutamine, aspartic acid with asparagine, arginine and alanine. All these amino acids are known as compounds storing nitrogen in vegetative storage organs of many plant species (McKee, 1962; Oland, 1959; Przybylska and Dąbrowska, 1970; Reuter, 1957; Wheeler and Bond, 1970).

The extensive investigations of Reuter (1957) involving 166 species belonging to 48 families demonstrated that in some species nitrogen storage in the form of common amino acids, while in other species in the form of nonprotein amino acids such as citrulline, azetidine-2-carboxylic acid or δ-acetylornithine. Some of the nonprotein amino acids are characteritic of a given species or genus. Our earlier studies on the composition of free amino acids in various organs of several perennial grass species (Rymowicz-Dąbrowska and Przybylska, 1970) demonstrated that in autumn glutamic acid with glutamine was dominant in the pool of free amino acids. These data and the present results indicate that in the vegetative storage organs of grasses, nitrogen storage is accomplished by common amino acids and amides. The nonprotein amino acids detected in tillering nodes and roots of *Dactylis glomerata*, such as γ-aminobutric acid, β-alanine and ornithine, occurred in small amounts, indicating that these compounds do not play any major role in nitrogen storage.

As indicated by the literature (McKee, 1962) most amino acids implicated in nitrogen storage in vegetative organs of plants are, at the same time, a form of nitrogen transport to the growing parts of plants at the onset of vegetation. The high concentration of glutamic acid with glutamine, aspartic acid and alanine in the spring period indicates that these compounds are, in this species, not only a form of storage, but also a form of nitrogen transport. Earlier data obtained for other grass species (Rymowicz-Dąbrowska and Przybylska, 1970) showed that the same compounds are active in nitrogen storage in vegetative organs and nitrogen transport to the growing tillers.

Noteworthly is the detection in tillering nodes and roots of *Dactylis glomerata* of γ-glutamyl-glutamine not found so far in the plant kingdom. γ-Glutamyl-glutamine concentration in the nodes and roots was low; its nitrogen constituted about 3 per cent of the soluble nitrogen of the organs examined.

Detection of γ-glutamyl-glutamine in *Dactylis glomerata* is interesting from yet another point of view. It is, to the author's knowledge, the first time that γ-glutamyl-peptide has been detected in grasses. Since that time about 30 compounds of this type have been revealed in plants. On the basis of the data accumulated to date, occurrence of γ-glutamyl-peptides is not correlated with the systematic classification of plants. Most of these compounds occur in species or genera far taxonomically distant.

The role of γ-glutamyl-peptides in the nitrogen metabolism of plants has so far not been clarified. In the light of certain data the function of storage or transport nitrogen may be attributed to them (Thompson, Morris, Arnold and Turner, 1962; Thompson, Turner and Gering, 1964).

3. Identification of some isolated free amino acids

From the pool of free amino acids in the tillering nodes and roots the following compounds have been isolated and obtained in crystalline form: asparagine, phenylalanine, aspartic acid, glutamic acid, tryptophane, histidine, arginine, lysine and two nonprotein amino acids — γ-aminobutyric acid and ornithine. The isolated compounds were identified by comparison with corresponding standard compounds as regards mobility on circular chromatograms, characteristic colour reactions, nitrogen content, IR absorption spectra, melting point and optical rotation. The analytical data for the isolated and corresponding standard amino acids are compiled in Table 1.

The isolation and full characterization of the amino acids isolated from tillering nodes and roots of *Dactylis glomerata* confirmes the identification based up on chromatographic data. It is worth stressing that research in which the common free amino acids detected in plant material are isolated in crystalline form, and then critically identified is extremely rare. Identification based up on chromatographic data is only tentative. To the author's knowledge, tryptophane has not yet been isolated from the free amino acids pool; the literature has described the isolation of this amino acid from protein hydrolysates (Greenstein and Winitz, 1961).

It is also worth noting the isolation in crystalline form of the nonprotein amino acid — ornithine. This compound has in general been

Table 1

Identification of isolated amino acids

Amino acids		Melting points °C	Optical rotation $[\alpha]_D^{23}$	Nitrogen content %
Glutaminic acid	A	211—213	+33.9° C, 0.58, 5n HCl	9.78
	B	207—208	+32.0° C, 0.5, 5 n HCl	9.52
Aspartic acid	A	271—272	+24.8° C, 0.2, 5n HCl	10.78
	B	270—271	+23.0° C, 0.5, 5n HCl	10.50
Asparagine	A	225	—5.4° C, 0.25, H_2O	18.5
	B	227	—5.0° C, 0.5, H_2O	19.0
Phenylalanine	A	283*	—7.4° C, 1.0, 5 nHCl	8.28
	B	282—284	—8.9° C, 1.0, 5n HCl	8.46
Tryptophane	A	285*	+5.7° C, 1.0, 1n HCl	13.52
	B	286*	+6.4° C, 1.0, 1n HCl	13.32
γ-Aminobutyric acid	A	203	0° C, 0.9, H_3O	13.58
	B	195	+0° C, 0.5, H_2O	13.46
Histidine	A	287*	+18,3° C, 1.0, 5n HCl	27.08
	B	286*	+17.5° C, 1.0, 5n HCl	27.1
Arginine	A	208—210	+21.8° C, 1.0, H_2O	32.16
	B	207—209	+20.0° C, 1.0, H_2O	31.5
Lysine 2HCl	A	200—202	+17.2° C, 1.0, 1n HCl	13.2
	B	200	+18.0° C, 1.0, 1n HCl	13.3
Ornithine 2HCl	A	145	+11.5° C, 1.0,H_2O	13.6
	B	143—145	+13.0° C, 1.0, H_2O	13.7

A — Standard L-amino acid; B — Isolated amino acid
* — Decomposition

identified in plant material on the basis of chromatographic data (McKee, 1962); it was isolated for the first time in crystalline form in 1958 from flax (*Linum usitarissimum*) (Coleman, 1958).

In summary it may be affirmed that precise analysis of the tillering nodes and roots of *Dactylis glomerata* has not revealed in this species the presence of specific nonprotein amino acids. γ-Aminobutyric acid, β-alanine and ornithine detected here are known to be common in the plant kingdom. These findings support the results of earlier investigations (Rymowicz-Dąbrowska and Przybylska, 1971) which did not reveal the presences in grasses of specific nonprotein amino acids.

The author wishes to express her cordial thanks to Docent dr J. Przybylska for her valuable guidance in the course of this work.

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Rozpuszczalne związki azotowe w węzłach krzewienia i korzeniach kupkówki pospolitej (Dactylis glomerata)

Streszczenie

Scharakteryzowano frakcję niskocząsteczkowych związków azotowych w węzłach krzewienia i korzeniach *Dactylis glomerata* w okresie wiosny i jesieni w ciągu trzech kolejnych lat rozwoju roślin.

Uzyskane dane wskazują, że w wegetatywnych organach spichrzowych kupkówki azot magazynowany jest przede wszystkim w postaci drobnocząsteczkowych związków azotowych. Spośród wolnych aminokwasów dużą rolę w magazynowaniu

azotu w węzłach krzewienia i korzeniach kupkówki odgrywają kwas glutaminowy z glutaminą, kwas asparaginowy, arginina i alanina. Należy zaznaczyć, że arganina gromadzona była głównie w węzłach krzewienia.

Wnikliwe badania nad frakcją rozpuszczalnych związków azotowych w węzłach krzewienia i korzeniach kupkówki nie wykazały obecności u tego gatunku charakterystycznych niebiałkowych aminokwasów. Wykryto tu γ-glutamylo-glutaminę, związek który nie był dotąd wykryty w świecie roślin.

Z puli wolnych aminokwasów węzłów krzewienia i korzeni wyizolowano w formie krystalicznej i jednoznacznie zidentyfikowano: kwas L-asparaginowy, L-asparaginę, kwas L-glutaminowy, L-fenyloalaninę, L-tryptofan, L-histydynę, L-argininę, L-lizynę, kwas γ-aminomasłowy oraz ornitynę, co stanowi bezpośrednie potwierdzenie identyfikacji wymienionych aminokwasów opartej o dane chromatograficzne.

Legend for Diagram 1

A — Total nitrogen, non-soluble nitrogen and soluble nitrogen (in per cent of dry matter)

B — Soluble nitrogen, α-amino nitrogen and amido nitrogen (in per cent of total nitrogen)

C — Total amido nitrogen, glutamine amido nitrogen, asparagine amido nitrogen (in per cent of total nitrogen)