JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2003,** *51,* 809−813 **809**

Flavone *C*-Glycoside, Phenolic Acid, and Nitrogen Contents in Leaves of Barley Subject to Organic Fertilization Treatments

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From the leaves of barley, *Hordeum vulgare*, one new flavone *C*-glucoside and three known flavone glucosides were isolated and characterized by ^{1}H and ^{13}C NMR and MALDI-TOF-MS. The novel flavone *C*-glucoside was isovitexin 7-*O*-β-[6‴-*O*-(*E*)-*p*-coumaroyl]glucoside (6‴-coumaroylsaponarin), and the known compounds were isovitexin 7-*O*-β-[6‴-*O*-(*E*)-feruloyl]glucoside, isoorientin 7-*O*-β-[6‴-*O*-(*E*)-feruloyl]glucoside, and tricin 7-*O*-β-glucoside. The sum of all the flavone glycosides and soluble phenolic acids in the leaves decreased with increased rate of plant nutrients given in animal manure and with increased crop yield. All of the major phenylpropanoids showed the same general response to nutrient level. The concentration of nitrogen in the leaves was not directly related to nutrient application or to contents of phenylpropanoids.

KEYWORDS: Barley; *Hordeum vulgare*; *C*-glycosyl flavones; phenolic acids; nitrogen; Askov Long-Term Experiments

INTRODUCTION

Many flavone *C*-glycosides have been identified in leaf extracts of *Hordeum* spp. (*1*−*11*), among these the novel 2′-hydroxyisoorientin (*12*). In this study, we report the structure of a new flavone *C*-glucoside, isovitexin 7-*O*-β-[6‴-*O*-(*E*)-*p*-coumaroyl]glucoside, along with those of three previously known flavone glycosides. Furthermore, we examine the relationships between levels of plant-available nutrients added in animal manure and the contents of phenylpropanoids and nitrogen in barley leaves. Laboratory studies of mainly young crop plants have shown that foliar concentrations of phenylpropanoids tend to decrease with increasing nutrient availability (*13*−*15*). Only a few studies have investigated this under field conditions (*16*). Various hypotheses attempt to explain the relationship between fertilization, plant growth rate, and the presence of phenolics in plant tissue, including the carbon−nutrient balance (CNB) hypothesis (*17*), the growth−differentiation balance (GDB) hypothesis (*18*), and the protein competition model (PCM) (*19*). They are all based on the concept that phenylpropanoids are involved in defense reactions of plants. Changes in phenylpropanoid concentrations may have implications for plant−pathogen interactions (*13*, *20*). This subject is of particular interest in organic farming, which relies heavily on natural plant defense mechanisms against fungal diseases. This study examines to what extent these relationships are valid for leaves from both young and mature barley plants grown under field conditions and amended with animal manure.

MATERIALS AND METHODS

Collection of Plant Material. For isolation of compounds, young field-grown plants of the winter barley cultivar Alexis were collected at the Danish Institute of Agricultural Sciences, Aarslev, in May 1998. No signs of leaf diseases were observed.

The effect of nutrient addition on leaf chemistry was studied on plant leaves retrieved from differently manured plots at the Lermarken site (B4-field) of the Askov Long-Term Experiments on Animal Manure and Mineral Fertilizers (*21*). These experiments were started in 1894 and grow a four-course rotation of winter cereals, root crops, spring-sown cereals, and grass/clover mixture established as an underseed in the spring-sown cereal crop. The B4-field has been managed organically without pesticides and mineral fertilizers since 1996. The treatments used in this study were unmanured plots (0) and plots taking different levels of nutrients (0.5, 1, 1.5; **Table 1**) in cattle slurry (SLU) or in cattle farmyard manure supplied with liquid manure (FYM). In the 1999 growth season, leaves of spring barley (*Hordeum vulgare* L. cv. Bartok) were sampled in the beginning of stem elongation (growth stage 30−32) (*22*) and at inflorescence emergence (growth stage 50−53). First and second leaves from the top (growth stage 30−32) and the two leaves just below the flag leaf (growth stage 50−53) were collected. No signs of leaf diseases were observed at growth stage 30−32, whereas

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10.1021/jf0258914 CCC: $25.00
Published on Web 12/21/2002

Table 1. Amounts of Nitrogen, Phosphorus, and Potassium in Farmyard Manure (FYM) and Cattle Slurry (SLU) Applied to *H. vulgare* Cv. Bartok, 1999

treatment code	N total (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})
0.0	0	0	0
0.5 FYM	50	15	31
1.0 FYM	100	29	62
1.5 FYM	150	44	93
0.5 SLU	50	10	40
1.0 SLU	100	20	79
1.5 SLU	150	30	119

minor infections were seen at growth stage 50−53 (<7.5% of the leaf area infected in any treatment).

Isolation of Flavonoids. Freeze-dried barley leaves (500 g) were extracted with 50% aqueous CH_3CN at room temperature for 1 h. The concentrated extract was adsorbed on an Amberlite XAD-7 column, washed with H_2O, and then eluted stepwise from 8 to 50% aqueous CH_3CN. The flavonoids **1**−**4** (**Figure 1**) were separated by preparative ODS-HPLC (20 ∅ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 35 °C using increasing amounts of CH_3CN (from 12 to 25%; flow rate = 7 mL min^{-1}; monitoring at 254 nm). Fractions that still contained more than one compound were separated by semi-preparative HPLC (LiChrospher 100 RP-18 (10 μm; 10 × 250 mm) at 35 °C in the same eluent system (flow rate = 3 mL min^{-1}). The pigment fractions were concentrated to dryness in vacuo and stored at −80 °C. From the leaves **1** (5 mg), **2** (8 mg), **3** (7 mg), and **4** (8 mg) were obtained.

HPLC Analysis. Freeze-dried leaves were extracted for 20 h with 40% acetonitrile at pH 3 (controlled with phosphoric acid). The filtered extract (30 μL) was analyzed by analytical HPLC using an RP-18 column (LiChrospher 100, 4 × 250 mm Nucleosil, Merck KGaA) at 35 °C. The elution profile was a linear gradient from 5 to 20% acetonitrile at pH 3 for 20 min, then from 20 to 30% for 5 min, from 30 to 40% for 2 min, and finally a linear gradient for 3 min from 40% back to 5% aqueous acetonitrile (pH 3, controlled by addition of H_3PO_4). The overall separation time was 40 min, including a stabilization period of 10 min with 5% acetonitrile (pH 3). Flow rate was 1.5 mL/min. The individual peak areas obtained at 280 nm (Shimadzu HPLC) were calculated in micromolar using concentration curves of apigenin and chlorogenic acid as standards, respectively.

Spectroscopic Analysis. UV−visible spectra were recorded in MeOH containing 0.1% HCl. Mass spectra were obtained by MALDI-TOF (Voyager-DE Pro) using a matrix of 2,5-dihydroxybenzoic acid in 10% EtOH. ^{13}C, HSQC, and HMBC spectra (JNM alpha 600, JEOL) were recorded in DMSO-d_6 with internal standard CD_2HSOCD_3 (2.50 ppm) at 110 °C, and ^{1}H, ^{1}H−^{1}H COSY, ^{1}H HOHAHA, and NOE difference spectra were measured at 23 °C, as described in ref *12*. ^{1}H HOHAHA and 2D spectra were obtained using a pulse sequence supplied from JEOL.

Measurements of Crop Biomass. For determination of root and shoot biomass whole plants were dug up by hand at growth stage 50−53. The plants were cleansed of soil using water and divided into roots and shoots (above-ground mass or the sum of all tillers on an individual plant). The plant material was dried at 80 °C to a constant weight (>24 h). Whole plot yields of grain and straw were determined at physiological maturity.

Measurements of Nitrogen. The contents of total N in freeze-dried leaf material were determined using a CN analyzer (Carlo Erba IT).

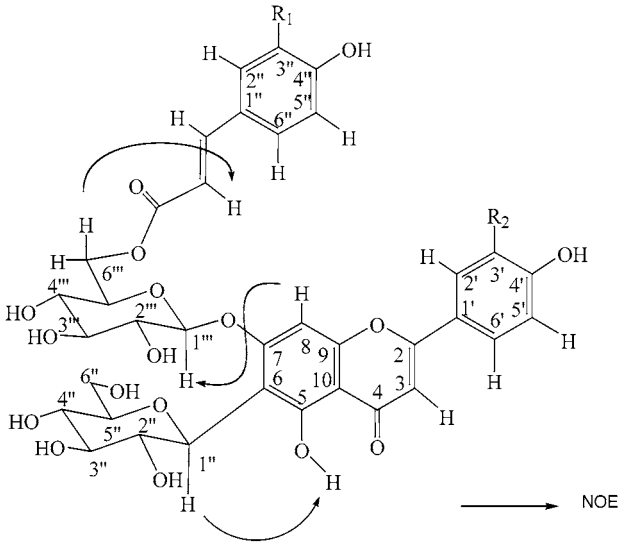
Estimation of Protein. Protein content was estimated by multiplying total-N content by a factor of 6.25 (*23*).

Statistical Analysis. Differences in contents of phenylpropanoids and N between the fertilization treatments were statistically analyzed using StatGraphics Plus 3.1 for variance analysis (ANOVA). Least significant difference was set at 5% (LSD_{95}).

RESULTS AND DISCUSSION

Four flavone glucosides were isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. Together with six flavonoids previously isolated (*12*) and two detected phenolic acids, the compounds were used as standards in a screening of phenylpropanoids in barley leaves treated with organic fertilization.

The UV absorption spectrum of **1** showed a flavone acylated by hydroxycinnamic acids (UV λ_{max} in MeOH, 236, 333sh, 350; + NaOMe, 234, 362; + $AlCl_3$, 242, 348sh, 398sh; + $AlCl_3$ + HCl, 242, 348sh, 398sh; +NaOAc, 254, 390; + NaOAc + H_3BO_3, 243, 384sh). The MALDI-TOF-MS spectrum of **1** showed a molecular ion at *m*/*z* 740. The ^{1}H NMR spectrum showed the presence of apigenin, two hexoses, and one cinnamoyl group (**Table 2**). Two anomeric protons were observed at δ_H 4.75 (d,



1: R_1= H, R_2 =H
2: R_1= OCH_3, R_2 = H
3: R_1= OCH_3, R_2 = OH

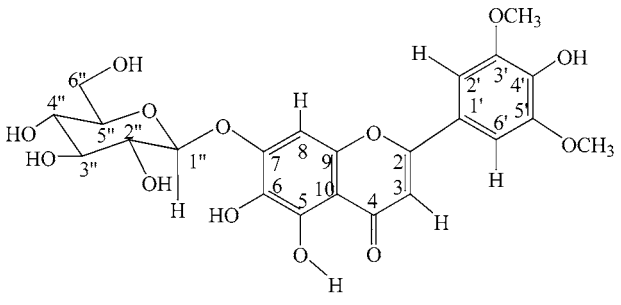


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Figure 1. Structures of isovitexin 7-*O*-β-[6‴-*O*-(*E*)-*p*-coumaroyl]glucoside (6‴-coumaroylsaponarin) (**1**), isovitexin 7-*O*-β-[6‴-*O*-(*E*)-*p*-feruloyl]glucoside (6‴-feruloylsaponarin) (**2**), isoorientin 7-*O*-β-[6‴-*O*-(*E*)-feruloyl]glucoside (**3**), and tricin 7-*O*-β-glucoside (**4**).

Table 2. ^{1}H NMR Chemical Shifts of Isovitexin 7-*O*-β-(6-*O*-Coumaroyl)glucoside, a Novel Flavone *C*-Glycoside Isolated from Young Leaves of Barley (DMSO-d_6; 600 MHz Instrument)

no.	^{1}H	no.	^{1}H
apigenin		6-*C*-glucoside (H″)	
		1″	4.75 d (9.6)
3	6.84 s	2″	4.04 t (8.8)
8	6.69 s	3″	3.32 (8.8)
2′	7.85 d (8.4)	4″	3.30 t (8.8)
3′	6.90 d (8.4)	5″	3.25 m
5′	6.90 d (8.4)	6″	3.68 dd (2.5; 11.8)
6′	7.85 d (8.4)	6″	3.58 m
coumaroyl		7-*O*-glucoside (H‴)	
CH=*CH*COO	6.26 d (15.6)	1‴	5.05 d (7.2)
CH=CHCOO	7.50 d (15.6)	2‴	3.46 t (8.8)
2″,6″	7.24 d (8.4)	3‴	3.42 t (8.8)
3″,5″	6.62 d (8.4)	4‴	3.34 t (8.8)
		5‴	3.84 m
		6‴	4.56 dd (2.4; 12.0)
		6‴	4.26 dd (6.6; 12.0)
5-OH[a]	13.41		

[a] α signal was broadening singlet.

J = 9.6 Hz, H-1″) and 5.05 (d, J = 7.2 Hz, H-1‴), and all vicinal coupling constants were 8.5−9.0 Hz (H-2″−H-4″, H-2‴−H-4‴), indicating *C*- and *O*-linked glucopyranosyl units, respectively (**Table 2**). The positions of the glucosidic linkages were determined by ^{1}H NOESY. NOE observed between OH-5 (δ 13.41) and H-1″ indicated a glucosyl (B) linkage to C-6. The glucosyl (J) moiety was attached to OH at C-7 from a negative NOE between H-8 (δ_H 6.69) and H-1‴ (**Figure 1**). The remaining signals in the ^{1}H NMR spectrum of **1** were identical to those of (*E*)-*p*-coumaric acid (**Table 2**). The 6-methylene protons of the J-glucoside were lowfield shifted by ∼0.8 ppm more than those of isovitexin 7-*O*-β-glucoside (*12*), indicating that the 6-OH of J-glucoside was acylated with the coumarate. This was confirmed by a weak NOE connectivity obtained between one of the 6‴-CH_2 moieties (δ_H 4.56) and the α-CH (δ_H 6.26) of the *p*-coumaric acid moiety. Thus, **1** is a new flavone, isovitexin 7-*O*-β-[6‴-*O*-(*E*)-*p*-coumaroyl]glucoside (6‴-coumaroylsaponarin) (**Figure 1**). ^{13}C NMR of **1** was not measured because of limited sample quantities available.

2 gave NMR data similar to those for **1**, except for a feruloyl substitute, which was confirmed by observation of a MALDI-TOF-MS peak due to the [M + H] ion at *m*/*z* 770 being 30 mass units larger than that of **1**. Thus, **2** is 6‴-feruloylsaponarin (**Figure 1**), which has earlier been isolated from barley leaves and identified by NMR (*8*).

The [M + H] of **3** at *m*/*z* 786 is 16 mass units larger than that of **2**. The chromophore proton signals were due to luteolin, whereas the signals of the sugar and phenolic acid were mutually identical to those of **2**. Thus, **3** was identified as isoorientin 7-*O*-β-[6‴-*O*-(*E*)-feruloyl]glucoside (**3**) (**Figure 1**), which previously has been documented by NMR data in barley leaves (*8*).

On the basis of spectroscopic data, compound **4** was identified as tricin 7-*O*-β-glucoside (**Figure 1**), which has been isolated from barley before (*4*, *8*). MS, ^{1}H NMR, and ^{13}C NMR data corresponded to the data in ref *24*.

Chlorogenic acid was identified by comparison of retention time on HPLC with authentic sample.

All of the major flavonoids identified (**Figure 2**) showed the same general response to nutrient level, and in the following the data are given for the sum of all flavonoids. Similarly, the phenolic acids are treated together. The contents of flavonoids in the leaves decreased significantly (**Figure 3**, values of $p \leq 0.05$) when the supply of plant nutrients increased, both for farmyard manure and for cattle slurry. Due to the presumed localization of flavonoids in the epidermis (*25*), the content is shown as micrograms per square centimeter of leaf surface. The concentrations of soluble phenolic acids in the leaves also decreased with increased addition of plant nutrients. At growth stage 30−32 the differences between treatments were small, without a clear trend, but differences became statistically significant for plants receiving both types of fertilizer at growth stage 50−53 (**Figure 4**, values of $p \leq 0.01$).

The amounts of total N in the leaves were not correlated with levels of nutrients applied, the unmanured plants accumulating high amounts of total N in the leaves (**Figure 5**), probably due to a relative deficiency of other mineral nutrients. Moreover, when nutrients are strongly limiting, moderate additions of fertilizer can result in greater yields of plant biomass, but with decreased protein concentrations. Severe nutrient deficiency is characterized by a poor growth rate, and root growth is restricted. Small additions of nutrients improve root performance and significantly improve the photosynthetic capacity of the above-ground biomass (*26*). The response of even small

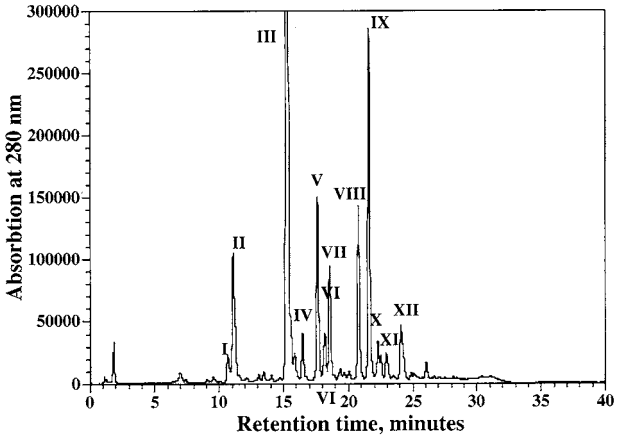

Figure 2. Chromatographic profile at 280 nm of the extract of barley leaves (*H. vulgare* L. cv. Bartok): I, unknown acid; II, chlorogenic acid; III, isoorientin 7-*O*-β-glucoside; IV, carlinoside; V, 2′-hydroxyisoorientin; VI, saponarin; VII, shaftoside; VIII, isoscoparin 7-*O*-β-glucoside; IX, isoorientin 7-*O*-β-(6-*O*-feruloyl)glucoside; X, 6‴-coumaroylsaponarin; XI, 6‴-feruloylsaponarin; XII, tricin 7-*O*-β-glucoside.

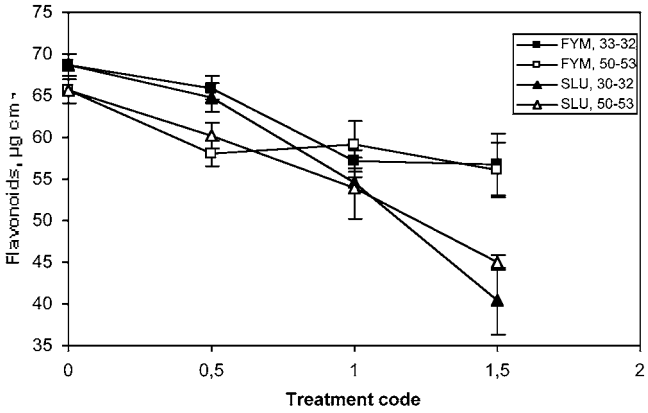

Figure 3. Flavonoids in the leaves of barley cv. Bartok at growth stages 30–32 and 50–53 (*22*) and fertilized with farmyard manure (FYM) or with cattle slurry (SLU). The decline with increasing fertilizer application is significant for both types of treatments at both growth stages. Means are given with standard errors. See **Table 1** for details on treatment code.

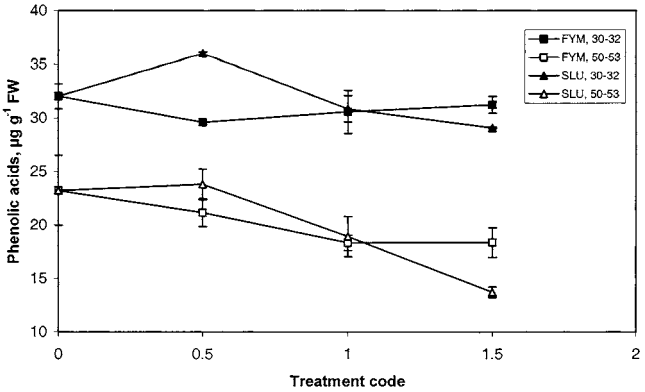

Figure 4. Phenolic acids in the leaves of spring barley *H. vulgare* cv. Bartok at growth stages 30–32 and 50–53 (*22*) and fertilized with farmyard manure (FYM) or with cattle slurry (SLU). The decline with increasing fertilizer application is significant for both types of manure at growth stage 50–53. Means are given with standard errors. See **Table 1** for details on treatment code.

additions of plant-available nitrogen can cause the nitrogen in the biomass to be diluted by a disproportionatly higher production of starch in the leaves, thereby reducing the nitrogen concentration. At higher levels of nitrogen addition, increases in nitrogen increase both biomass yield and protein concentrations (**Table 3**). Thus, the barley responded to fertilization by increasing biomass production and yield and by showing different allocation patterns, in terms of root-to-shoot ratio and straw and grain weights (**Tables 4** and **5**).

Several partially overlapping hypotheses have been proposed to predict a relationship between nutrient availability, plant growth rate, and allocation to phenolics. The carbon−nutrient balance (CNB) hypothesis makes predictions about the relationship between the carbon/nutrients ratio of the plant and allocation to phenolics versus N-containing secondary metabolites (*17*). Limited availability of nutrients will reduce growth (biomass accumulation) more than photosynthesis, and a surplus of nonstructural carbohydrates will accumulate. These will be diverted into an enhanced production of carbon-based secondary metabolites, for example, phenylpropanoids. The growth−differentiation balance (GDB) hypothesis suggests that biomass accumulation and secondary chemistry are negatively correlated

Table 3. Estimation of Protein Concentrations in Leaves, Straw, and Grain of Spring Barley (Cv. Bartok) Supplied with Levels of Farmyard Manure (FYM) or Cattle Slurry (SLU)[a]

	protein (mg/g of dw)		
treatment code	leaves, growth stage 50–53	straw, physiological maturity	grain, physiological maturity
0.0	131.4 (9.7)	93.1 (4.7)	82.5 (3.1)
0.5 FYM	118.4 (4.9)	110.6 (0.6)	77.8 (0.9)
1.0 FYM	125.8 (7.7)	102.1 (2.1)	80.8 (2.4)
1.5 FYM	133.3 (12.0)	100.6 (1.3)	78.3 (3.3)
0.5 SLU	118.0 (2.6)	91.3 (13.8)	78.8 (3.8)
1.0 SLU	143.6 (4.4)	71.3 (15.6)	77.8 (1.6)
1.5 SLU	156.1 (15.4)	54.7 (0.3)	83.4 (0.3)

[a] See **Table 1** for details on treatment code. Means are given with standard errors in parentheses.

Table 4. Biomass of Roots and Shoots (Comprising All Above-Ground Biomass on an Individual Plant) of Spring Barley (Cv. Bartok) at Growth Stage 50–53[a]

treatment code	root dry mass (g)	shoot dry mass (g)	root/shoot ratio
0.0	0.11 (0.01)	0.49 (0.02)	0.22 (0.02)
0.5 FYM	0.18 (0.03)	0.85 (0.09)	0.21 (0.04)
1.0 FYM	0.32 (0.03)	1.54 (0.12)	0.21 (0.03)
1.5 FYM	0.21 (0.04)	1.52 (0.18)	0.13 (0.01)
0.5 SLU	0.15 (0.02)	0.82 (0.09)	0.19 (0.03)
1.0 SLU	0.15 (0.01)	1.09 (0.10)	0.14 (0.01)
1.5 SLU	0.20 (0.04)	1.38 (0.11)	0.15 (0.04)

[a] Means are given with standard errors in parentheses.

(*18*). In plants growing at adequate to high nutrient availability, resources will preferentially be shunted into growth processes, resulting in a lower production of secondary metabolites. The protein competition model (PCM) postulates a more specific competition between primary and secondary pathways of metabolism. In a period of rapid plant growth, as in the case of high nutrient availability, phenylalanine preferentially flows into protein synthesis rather than toward synthesis of phenylpropanoids via phenylalanine ammonia lyase (*19*).

The present study is in accordance with the core of these overlapping hypotheses, because the concentrations of both types of phenylpropanoids tend to decrease with increasing nutrient

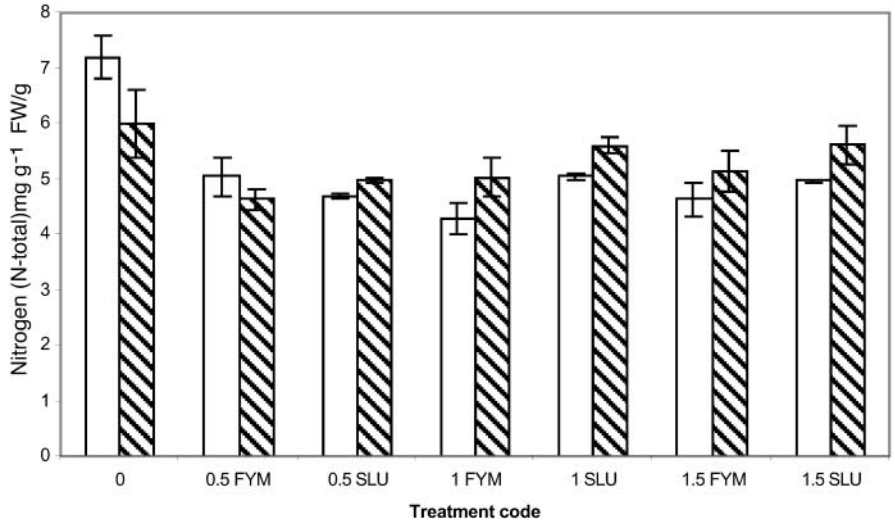

Figure 5. Nitrogen concentrations (total N) in the leaves of spring barley (cv. Bartok) supplied with different levels of farmyard manure (FYM) or cattle slurry (SLU): white bars, growth stage 30–32; ruled bars, growth stage 50–53. Means are given with standard errors. See **Table 1** for details on treatment code.

Table 5. Yield of Straw and Grain of Spring Barley (Cv. Bartok) at Physiological Maturity[a]

treatment code	straw (t dw ha^{-1})	grain (t dw ha^{-1})	grain/straw ratio
0.0	0.63 (0.09)	0.86 (0.05)	1.40 (0.13)
0.5 FYM	2.11 (0.33)	1.27 (0.13)	0.63 (0.16)
1.0 FYM	2.71 (0.32)	1.82 (0.01)	0.69 (0.08)
1.5 FYM	3.30 (0.36)	2.12 (0.04)	0.66 (0.10)
0.5 SLU	1.86 (0.08)	1.36 (0.03)	0.73 (0.05)
1.0 SLU	2.93 (0.10)	2.32 (0.26)	0.80 (0.11)
1.5 SLU	3.43 (0.51)	3.34 (0.23)	0.98 (0.08)

[a] Means are given with standard errors in parentheses.

availability and biomass production. However, the unmanured plant leaves were high in both nitrogen and phenylpropanoids, and this conflicts with the CNB hypothesis, if the carbon/nutrient ratio of the plant is based on the ratio between carbon and nitrogen. However, if the carbon/nutrient ratio of the plant is based on nutrients other than nitrogen, we cannot reject the CNB hypothesis, because the unmanured plants may be limited by nutrients other than nitrogen. The hypotheses were developed primarily to explain the composition of perennial species, where differentiation (production of secondary metabolites) is expressed in terms of increased longevity and can be differentiated from growth in terms of, for example, wood density. This is not applicable for an annual plant such as barley with relatively low contents of phenylpropanoids, compared to perennial species. Still, it was observed in the field that senescence was slightly delayed for treatments that received no or little fertilizer. This is also reflected in the temporal change in soluble phenolic acid content, which declined more rapidly for the well-fertilized plants (**Figure 4**). However, provided that rapid growth is reflected in increased production of plant biomass, our data support the GDB hypothesis as the contents of both flavonoids and, at the later growth stage, phenolic acids decrease with increasing biomass accumulation. The relatively high contents of both phenylpropanoids and protein in the unmanured leaves could indicate a discrepancy with the PCM hypothesis. However, the very low amounts of phenylpropanoids in the leaves show that the demand for phenolics does not limit protein synthesis. It should also be noted that other stress factors not measured in this study, for example, high light/UV, can have an effect on the synthesis of phenylpropanoids (*27*). The implications of the present findings for disease susceptibility will be presented in a separate paper.

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Received for review August 15, 2002. Accepted November 26, 2002. This study was financially supported by the Danish Research Centre for Organic Farming (DARCOF) (http://www.agrsci.dk/foejo/GB/sider/darcof/index_darcof.html).

JF0258914