JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Effect of Fertilizers on Galanthamine and Metabolite Profiles in *Narcissus* Bulbs by ^{1}H NMR

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ABSTRACT: *Narcissus* bulbs contain the biologically active alkaloid galanthamine, and *Narcissus* is being developed as a natural source of the molecule for the pharmaceutical industry. The effect of fertilizer on galanthamine production was investigated in a field study using a ^{1}H nuclear magnetic resonance (NMR) metabolite profiling approach. Galanthamine was quantitated and major metabolites in the bulbs were identified. The application of standard fertilization levels of nitrogen and potassium caused a significant increase in galanthamine as compared to a control. Multivariate data analysis of the ^{1}H NMR data revealed that applying double the standard level of nitrogen fertilizer resulted in production of more amino acids and citric acid cycle intermediates, but not more galanthamine. The results indicated that standard levels of fertilizer currently applied in The Netherlands are sufficient for optimal galanthamine accumulation in the bulbs. This study shows how ^{1}H NMR-based metabolic profiling can provide insight into the response of plant metabolism to agricultural practices.

KEYWORDS: galanthamine, *Narcissus pseudonarcissus*, fertilizer, ^{1}H NMR, metabolic profiling

INTRODUCTION

Galanthamine (Figure 1) is a benzazepine alkaloid produced by Amaryllidaceae plants. In 1952 it was first isolated from *Galanthus woronowii*, and has also been found in members of the genera *Amaryllis*, *Lycoris*, *Leucojum*, and *Narcissus*. Galanthamine has been pharmacologically investigated since its discovery and numerous biological activities have been found.[1] Its long-lasting, selective, reversible and competitive inhibition of the enzyme acetylcholinesterase attracted much attention and lead to its application to Alzheimer's disease. Galanthamine has been registered as a drug (Nivalin, Reminyl and later Razadine) since the mid-1990s and is used for relief of the symptoms of Alzheimer's disease.[2]

Galanthamine can be produced synthetically, but extraction from plants is still the main source for the pharmaceutical industry. Currently galanthamine is extracted from *Narcissus* cultivars, *Leucojum aestivum*, *Lycoris radiata*, and *Ungernia victoria* in different parts of the world.[3] In The Netherlands *Narcissus pseudonarcissus* L. is under development as a source for galanthamine production. The cultivar "Carlton" was chosen as it contains galanthamine as the major alkaloid in the bulbs, large stocks of bulbs are already commercially available and cultivation practices are well established due to the long tradition of ornamental use.[4]

The effect of nitrogen fertilization has been studied in various alkaloid-producing plants. Since alkaloids contain nitrogen and are usually derived from amino acid precursors, it has generally been assumed that higher levels of nitrogen availability would lead to higher levels of alkaloids in plants.[5] This was shown to be the case in studies done in the 1970s on crops such as tobacco (*Nicotiana* spp.), lupines (*Lupinus* ssp.), barley (*Hordeum vulgare*), *Atropa*, *Papaver*, and *Datura*.[6] In more recent studies for example on periwinkle (*Catharanthus roseus*), yaupon

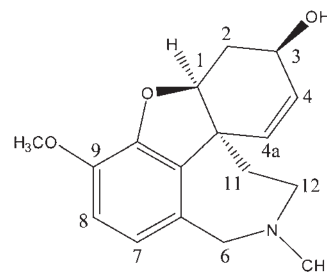

Figure 1. Chemical structure of galanthamine.

(*Ilex vomitoria*), and *Datura* increased alkaloid accumulation was also observed as a result of nitrogen fertilization.[7−9]

In *N. pseudonarcissus* 'Carlton' fertilization increased galanthamine content in the bulbs as compared to no fertilization.[4] We wanted to know whether the standard level of fertilization as typically applied to increase the yield of ornamental *Narcissus* crops in The Netherlands is optimal for galanthamine production, and whether adding more fertilizers would lead to more galanthamine accumulating in the bulbs. In the study of Kreh[4] galanthamine was determined after the bulbs were in the ground for two years. In The Netherlands bulbs are usually lifted after one year, so it was also of interest to determine fertilizer effects on galanthamine within the typical time frame of cultivation.

Many methods have been published for the determination of galanthamine in plant material.[10] Most of these are chromatography-based methods that involve selective alkaloid extraction

Received: November 17, 2010
Accepted: February 15, 2011
Revised: February 12, 2011
Published: March 04, 2011

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and analysis requiring an analytical standard. Such methods can be used to compare levels of a compound between treatments in an agricultural experiment. To determine why a particular metabolite level is changed a broader view of the plant metabolism is needed.

In this study galanthamine in *N. pseudonarcissus* bulbs treated with different levels of fertilizer was determined using a quantitative proton nuclear magnetic resonance (^{1}H NMR) method developed previously.[11] The NMR spectra were analyzed by principal component analysis (PCA) to observe effects on metabolite profiles other than changes in galanthamine. A targeted approach using gas chromatography (GC) was also used to assess relative ratios of alkaloids in the bulbs.

MATERIALS AND METHODS

Fertilizer Treatments. Bulbs of *Narcissus pseudonarcissus* (cv. Carlton) were planted in November 2008 in sandy soil in Lisse, The Netherlands. For each treatment a plot of bulbs was planted, which consisted of two rows of 11, and two rows of 10 bulbs (total 42 bulbs). The rows were planted 18 cm apart, and each plot was surrounded by an edge of open space of about 70 cm. Fertilizers were applied in the spring, and one plot was left untreated for a control. The standard nitrogen application was 110 kg/hectare of Kalksalpeter ($Ca(NO_3)_2$)with 19% Ca and 15.5% N, consisting of 14.4% *N*-NO_3, 1.1% *N*-NH_4) applied in three applications of 40, 40, and 30 kg applied on 12 March, 26 March, and 29 April, respectively. The double nitrogen treatment received an additional 40, 40, and 30 kg of nitrogen fertilizer on 19 March, 2 April, and 5 May, respectively. The standard potassium treatment consisted of 150 kg of Patentkali (K_2SO_4 and $MgSO_4$, 30% K as K_2O, 10% Mg as MgO and 42% S as SO_3) given in two applications of 75 kg each on 12 March and 26 March. The double potassium treatment received two extra applications of 75 kg each on 19 March and 2 April. The bulbs did not receive a hot water treatment and were not disinfected before planting. No herbicides, fungicides or insecticides were used on the field. The bulbs were lifted at the beginning of July and were dried and stored for two weeks in the shed at 20 °C before analysis. Bulbs were inspected and 10 healthy bulbs showing no fungal rot or other disease symptoms were selected from each treatment for analysis.

^{1}H NMR Analysis. *Extraction.* Plant material was prepared for NMR analysis according to the method of Kim et al.[12] Bulbs were dusted and rinsed to remove soil particles. Roots and basal plate was removed to aid cutting. Bulbs were frozen in liquid nitrogen and individually ground to fine powder in a Waring laboratory blender (Waring Products Inc., Torrington, CT). Ground bulb material was freeze-dried for 7 days. Fifty milligram of freeze-dried bulb material was weighed into 2 mL microtube and extracted with a mixture of 0.75 mL methanol-d_4 (99.80% from Cambridge Isotope Laboratories, Andover, MA), and 0.75 mL phosphate (KH_2PO_4) buffer (pH 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylpropionic acid sodium salt-d_4 (TMSP, w/w) as an internal standard for quantitation and calibration of chemical shift. Samples were ultrasonicated for 30 min, followed by centrifugation at 13 000 rpm for 10 min. An aliquot of 1 mL of the supernatant was collected and 800 μL transferred to 5 mm NMR tubes for ^{1}H NMR measurement.

Measurement. ^{1}H NMR measurements were carried out as described Kim et al.[13] ^{1}H NMR spectra were recorded with a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample 64 scans were recorded using the following parameters: 0.167 Hz/point and pulse width (PW) 4.0 μs. For accurate quantitation of galanthamine a relaxation delay (RD) of 5.0 s was used. FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to 0.0 ppm.

Data Analysis. For quantitative analysis of galanthamine, integration of the proton signal at δ 6.17 (galanthamine H-4a) was performed. The ratio of this integral to that of the internal standard was used to calculate the amount of galanthamine per milligram material. For multivariate data analysis, ^{1}H NMR spectra were automatically binned by AMIX software (v.3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32−10.0 was reduced to integrated regions ("buckets" or bins) of 0.04 ppm each. The regions δ 4.7−5.0 and δ 3.30−3.34 were excluded from the analysis because of the presence of the residual water and methanol signal, respectively. Principal component analysis (PCA) was performed with SIMCA-P$^+$ software (v. 12.0 Umetrics, Umeå, Sweden) using the Pareto scaling method. Quantitation of galanthamine was performed using Sigma Plot version 11.0. A one-way analysis of variance (ANOVA) followed by a pairwise multiple comparison (Tukey test) was used for comparing the average galanthamine levels between treatments. ANOVA of all NMR signal "buckets" was performed using Multiexperiment Viewer (v.4).[14]

Gas Chromatography. *Extraction.* Bulb material was extracted using the method described by Bastos et al.[15] Five hundred mg freeze-dried bulb material (as prepared for ^{1}H NMR measurements) was extracted in 6 mL of 0.05 N HCl solution by shaking for 2 h. The extracts were centrifuged for 5 min at 3500 rpm. Three mL of the supernatant was collected, and this was basified by adding 1 mL of a 0.3 N NaOH solution. Three mL of chloroform was added and the solution mixed by vortexing for 1 min. The solution was centrifuged for 5 min at 45 000 rpm, and the organic (lower) chloroform layer was collected. After washing the organic layer through a Pasteur pipet containing anhydrous magnesium sulfate, 1.5 mL was transferred to a test tube and dried under vacuum in a Savant speedvac (Thermo Scientific, Waltham, MA). The dried extracts were redissolved in 100 μL methanol containing 0.25 mg/mL papaverine (BDH laboratory reagents, Poole, England) as internal standard.

Measurement. Gas chromatography was carried out on an Agilent 6890 gas chromatographer with a FID detector and Agilent 7683 Series Injector (Agilent Technologies, Inc., Santa Clara, CA). The GC column used was a 30 m × 0.25 mm i.d., 0.25 μm film thickness CB-5 column (Agilent JW Scientific, Agilent Technologies, Inc., Santa Clara, CA). Helium was used as carrier gas with a flow rate of 1.5 mL/min. The oven temperature was programmed as follows: 200−250 at 2.5 °C/min, followed by increase of 250−270 at 10 °C/min, held at 270 °C for 8 min. The total run time was 30 min. The injector and detector temperatures were 250 and 270 °C, respectively. Four μL of the samples were injected, with the injector operating in split mode (1/20). GC-MS was carried out on an Agilent 7890A GC system with a 5975C single quadrupole Mass Spectrometric Detector and an Agilent 7693 Autosampler (Agilent Technologies, Inc.). The GC column used was a 30 m × 0.25 mm i.d., 0.25 μm film thickness HP-5 column (Agilent JW Scientific, Agilent Technologies, Inc.). The flow rate, oven temperature program and injector parameters were the same as for the FID analyses. The analysis was done in scan mode (m/z 50−350) using electron ionization at 70 eV.

RESULTS AND DISCUSSION

Galanthamine content in *N. pseudonarcissus* bulbs was determined using the quantitative ^{1}H NMR method described in Lubbe et al.[11] The average galanthamine levels (mg/g DW) was calculated, and is shown in Figure 2. The average galanthamine level of the control treatment was 2.92 ± 0.22 mg/g DW. The samples treated with the standard N and K application had an average galanthamine content which was significantly higher (P < 0.05) than the control. The other two treatments had slightly higher galanthamine levels on average, but these were not statistically different from the control.

Alkaloid extracts of the bulbs were analyzed by GC-FID to determine the relative amounts of alkaloids present. GC-MS was used for identification of the alkaloid peaks in the chromatograms (Table 1). The results of the GC analysis are shown in Figure 3. The fertilizer treatments altered the amount of alkaloids present, but the pattern of alkaloids was unchanged.

Inspection of the ^{1}H NMR spectra showed the presence of the same metabolites in all the bulb samples, but with relative amounts differing between treatments (Figure 4). Major signals in the ^{1}H NMR spectra were assigned to primary metabolites such as sucrose, citric acid, fatty acids, and various amino acids (Table 2). Signals belonging to galanthamine and hemeanthamine, the two most abundant alkaloids in this cultivar of *N. pseudonarcissus* were also identified. Signal assignments in this NMR solvent system matched those previously reported [11,17,18] and were confirmed using an in-house-built database of ^{1}H NMR spectra of metabolites from plants.

In Figure 4 the control sample spectrum (A) contains the same major metabolite signals as the treatments, but with the exception of sucrose they are less intense. In the standard nitrogen and potassium treatment (B) signals assigned to galanthamine as well and other signals in the aromatic region (hemeanthamine, *cis*-aconitic acid, 4-hydroxyphenylpyruvate) and fatty acid signals were increased compared to the control. These signals were also higher than the control in the double nitrogen and standard potassium treatment (C), but appeared to be smaller than in 4B. Amino acid signals from 3.3 to 1.5 ppm were clearly higher than in any of the other treatments. In 4D (standard nitrogen and double potassium) most metabolite signals were also higher than the control treatment, but galanthamine and other signals in the aromatic region were smaller than the standard nitrogen and potassium treatments. The amino acid signals from 3.3 to 1.5 were not as high as in 4C but appeared to be slightly higher than in the standard fertilizer treatment.

The differences in signal intensity between treatments were not easy to judge by visual inspection. NMR spectra can give useful qualitative and quantitative information about a sample in a single measurement, without being compound class selective.[19] To compare the intensities of many NMR signals between different spectra simultaneously, multivariate data analysis can be used. After acquiring the spectra, they are processed to extract the data. One way is through "binning" or "bucketing", in which spectra are split into discrete regions and integrated.[20] Multivariate data analysis methods such as principal component analysis (PCA) can be used to visualize the data. PCA compares all data "buckets" (variables) between samples simultaneously. This enables clustering of samples to reduce the dimensionality

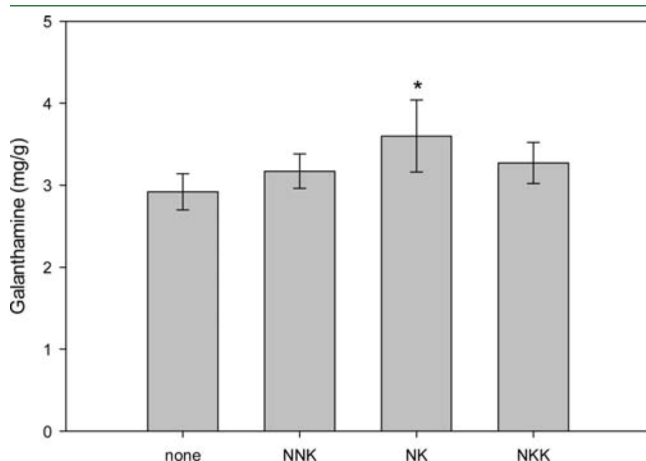

Figure 2. Average galanthamine levels of *Narcissus* bulbs treated with fertilizers compared to bulbs treated with no fertilizer (n = 10), determined by quantitative NMR analysis. NNK: double nitrogen, standard potassium; NK: standard nitrogen and potassium; NKK: standard nitrogen, double potassium. *Significant at $P < 0.05$.

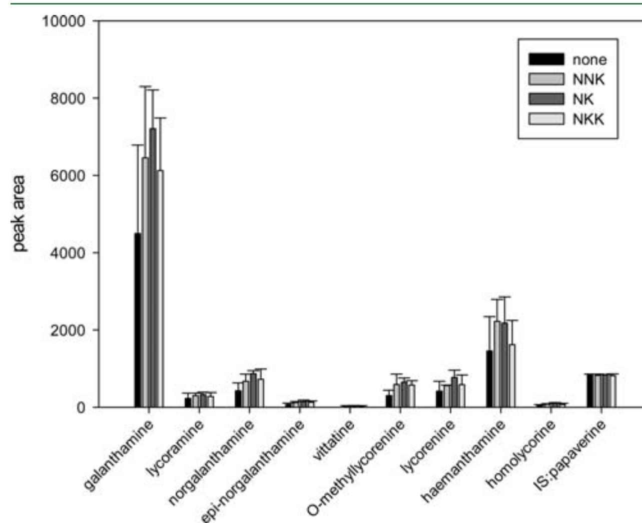

Figure 3. Average peak areas of alkaloids in GC-FID chromatograms of *Narcissus* bulbs treated with different levels of fertilizers. None: no fertilizer treatment, NNK: double nitrogen, standard potassium; NK: standard nitrogen and potassium; NKK: standard nitrogen, double potassium. IS: internal standard (papaverine).

Table 1. GC-MS Ion Fragmentation Patterns and Retention Times of Alkaloids in Extracts of *Narcissus* Bulbs

alkaloid	RT (min)	$[M]^+$ and characteristic ions (% compared to base peak)
galanthamine	12.78	287 (83), 286 (100), 244 (24), 216 (33), 174 (28)
lycoramine	13.00	289 (59), 288 (100), 232 (7), 202 (10), 115 (11)
norgalanthamine	13.42	273 (99), 272 (100), 230 (35), 202 (28), 174 (13)
epi-norgalanthamine	13.72	275 (78), 274 (100), 202 (11), 188 (18), 175 (12)
vittatine	13.98	271 (100), 228 (22), 199 (71), 187 (66), 115 (38), 56 (18)
O-methyllycorenine	14.71	331 (<1), 300 (3), 191 (8), 109 (100)
lycorenine	17.21	317 (<1), 299 (6), 191 (1), 179 (1), 109 (100), 94 (3)
hemeanthamine[a]	17.82	301 (13), 272 (100), 240 (16), 211 (14), 181 (24)
homolycorine	20.69	315 (<1), 281 (2), 207 (7), 178 (2), 109 (100), 94 (3), 82 (3)

[a] Decomposition product of hemeanthamine, Kreh et al.[16]

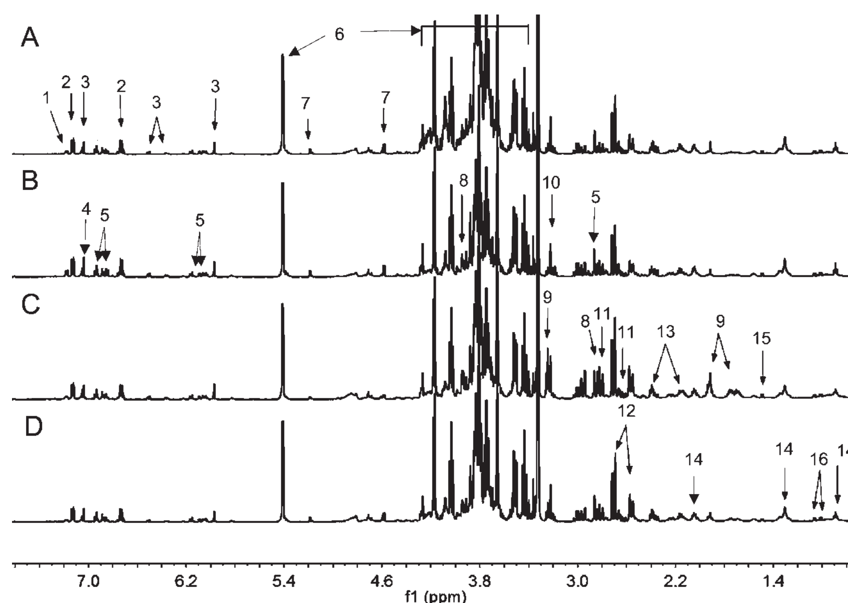

Figure 4. ^{1}H NMR spectra from 7.6 to 0.8 ppm of bulbs treated with (A) no fertilizer, (B) standard nitrogen and potassium, (C) double nitrogen and standard potassium, and (D) standard nitrogen, double potassium. 1: tyrosine, 2: 4-hydroxyphenylpyruvate, 3: hemeanthamine, 4: *cis*-aconitic acid, 5: galanthamine, 6: sucrose, 7: glucose, 8: asparagine, 9: ornithine, 10: choline, 11: aspartic acid, 12: citric acid, 13: glutamic acid, 14: fatty acid, 15: alanine, 16: valine.

Table 2. ^{1}**H Chemical Shifts (δ) and Coupling Constants (Hz) of *N. pseudonarcissus* Metabolites in Methanol-d_4-KH_2PO_4 in D_2O at pH 6.0**

metabolite	chemical shift (δ) and coupling constant (Hz)
tyrosine	7.18 (d) J=8.4, 6.85 (d) J=8.4
4-hydroxyphenylpyruvate	7.13 (d) J=8.4, 6.73 (d) J=8.4, 3.02 (d) J=13.6, 2.98 (d) J=13.6
cis-aconitic acid	7.04 (s)
galanthamine	6.94 (d) J=8.3, 6.88 (d) J=8.3, 6.16 (d) J=10.5, 6.06 (dd) J= 10.5, 5.0, 2.86 (s)
hemeanthamine	7.06 (s), 6.71 (s), 6.51 (d) J=10.3, 6.36 (dd) J=10.3, 5.0, 5.97 (brs)
sucrose	5.41 (d) J=3.8, 4.17 (d) J=8.7, 4.03 (t) J=8.3, 3.78−3.83 (m), 3.75 (t) J=9.5, 3.66 (s), 3.51 (dd) J=9.9, 3.9, 3.43 (t) J=9.5
glucose	4.58 (d) J=7.9, 5.19 (d) J=3.8, 3.20 (dd) J=8.8, 8.9
choline	3.21 (s)
asparagine	3.94 (dd) J=8.0, 4.0, 2.95 (dd) J=17.0, 3.8, 2.81 (dd) J=17.0, 8.2
aspartic acid	2.82 (dd) 17.0, 8.5, 2.63 (dd) J=17.0, 9.5
citric acid	2.71 (d) J=15.8, 2.56 (d) J=15.8
glutamic acid	2.39 (td) J=7.1, 2.5, 2.10−1.28 (m)
ornithine	3.24 (t) J=7.0, 1.92 (m), 1.65−1.78 (m), 3.71 (t) J=5.8
alanine	1.49 (d) J=7.2
valine	1.06 (d) J=7.0, 1.01 (d) J=7.04
fatty acid	1.31 (brs), 0.89 (t) J=7.1, 5.40 (m)

of the data set and reduce the number of variables needed to describe it.[21] Through further analysis the spectral areas responsible for grouping in the samples can be investigated, and responsible compounds can be identified.

In this study PCA was used to reduce the complexity of the ^{1}H NMR data and see what caused the maximum variance between the samples. A seven-component model explained 94.2% of the variance, with the first two components explaining 80.5%. In the score plot of PC1 and PC2 in Figure 5A, the control samples were clearly separated from the treated samples along PC1. The samples with the lowest score on PC1 (most to the left on score plot) were those treated with double nitrogen (NNK). Samples treated with the standard nitrogen (NK) had PC1 scores intermediate between control and double nitrogen (NNK). Double nitrogen (NNK) treatments were separated from the standard nitrogen and potassium (NK) and double potassium (NKK) treatments along PC2. Samples treated with double potassium (NKK) had scores similar to the standard nitrogen and potassium (NK) samples, and were not separated from the other treatments along any of the major principal components.

A loading scatter plot of PC1 vs PC2 (Figure 5B) shows how different areas of the spectrum contribute to the grouping of samples seen in the PCA score plot. The signals important for discriminating the control samples belong to sucrose. The signals

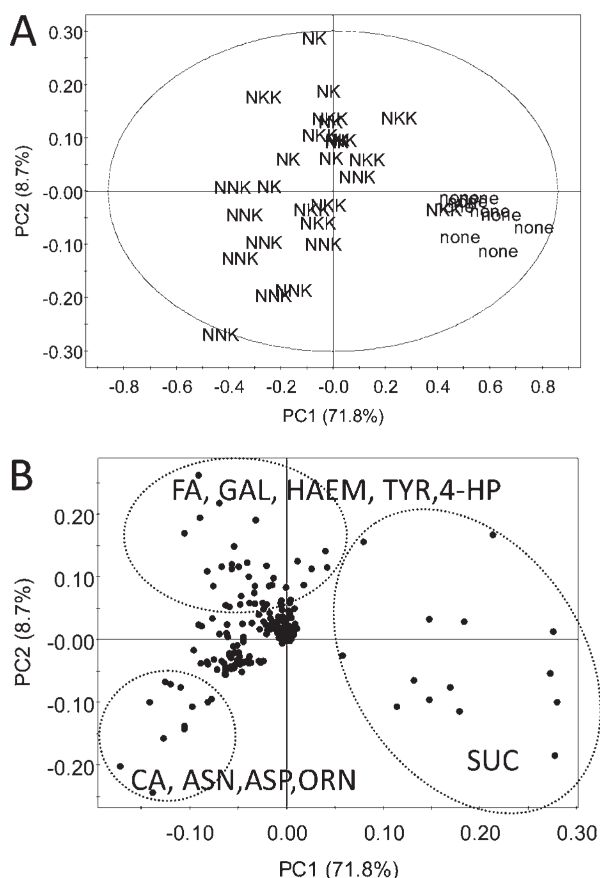

Figure 5. (A) Score plot of PCA (PC1 vs PC2) of *Narcissus* bulbs treated with different levels of fertilizers. The elipse represents Hotelling's T2 with 95% confidence. None: no treatment; NK: standard nitrogen and potassium; NNK: double nitrogen, standard potassium; NKK: standard nitrogen, double potassium. (B) Loading scatter plot of PCA (PC1vs PC2) of *Narcissus* bulbs treated with different levels of fertilizers. The control (none) region of the loading plot contains NMR signals assigned to sucrose. The standard nitrogen and potassium (NK) region of the plot contains signals of galanthamine (GAL), hemeanthamine (HAEM), fatty acids (FA), tyrosine (TYR) and 4-hydroxyphenylpyruvate (4-HP). The double nitrogen and standard potassium (NNK) region of the plot contains signals of citric acid (CA), as well as the signals of aspartic acid (ASP), asparagine (ASN) and ornithine (ORN).

associated with the double nitrogen treatment were mostly below δ 3.0. These signals were assigned to asparagine, aspartic acid, citric acid, and ornithine. For samples that received the standard nitrogen application, ^{1}H NMR signals responsible for their position on the score plot were mostly at higher values, between δ 5.0 and δ 7.5. Signals responsible for high scores along PC2 were galanthamine, hemeanthamine, tyrosine, 4-hydroxyphenylpyruvate, and fatty acid signals.

To determine which of the signals contributing to the PCA model were statistically different between treatments, ANOVA was performed on the integrated regions (buckets) of the NMR spectra. Figure 6 shows the results of the ANOVA, which confirms what was seen in the PCA.

Optimizing the yield of galanthamine from *Narcissus* bulbs is desirable for the economically feasible extraction of this compound for pharmaceutical use. While studies have focused on increasing galanthamine production in cell or tissue culture systems,[22] less is known about this in field situations. Kreh[4] described a field test to assess the effect of fertilizers on the galanthamine content of *Narcissus* 'Carlton'. It was found that applying either nitrogen or potassium/magnesium fertilizer significantly increased the level of galanthamine in the bulbs compared to an untreated control. Since fertilizer was applied at one level, it is not known whether applying additional fertilizer would result in a further increase of galanthamine.

In this study, galanthamine in *N. pseudonarcissus* bulbs was lowest when the plants were not treated with fertilizer. Compared to no treatment, the standard application of nitrogen and potassium resulted in significantly higher levels of galanthamine. Double application of nitrogen and potassium both resulted in a higher level of galanthamine on average, though the difference was not statistically significant compared to the control. Alkaloid extraction and GC analysis revealed that the other minor alkaloids in the bulbs followed the same pattern, that is, lowest levels occurred in the no fertilizer treatments, highest in the standard treatments and intermediate in the double nitrogen or potassium treatments.

The spectra of control bulbs that received no fertilizer were dominated by sucrose, which was has previously been reported as the major free sugar in *Narcissus* bulb scales.[24] Nitrogen assimilation from the soil consumes carbon compounds produced from assimilated CO_2. Since less nitrogen was available to the control bulbs, it appears that more photosynthetic products were stored in the bulbs as sucrose. In the bulbs that received standard nitrogen fertilization, the availability of nitrogen throughout the season caused increased production of amino acids, fatty acids, and alkaloids. Biosynthetic precursors to the alkaloids, tyrosine, and 4-hydroxyphenylpyruvate were also increased. The double nitrogen fertilizer treatment resulted in bulbs with more amino acids and citric acid than the other treatments. Increases in arginine and asparagine were previously reported in roots of *N. pseudonarcissus* upon nitrogen fertilization,[25] and was here also seen in the bulbs. The levels of alkaloids in the double N treatment were on average less than that of the standard nitrogen, although it was not significantly lower. The higher levels of amino acids indicate that the plants metabolism shifted toward producing more amino acids instead of producing more alkaloids. This is also reflected in the higher citric acid content, which is involved in the interconversion of amino acids and providing of precursors for many biosynthetic pathways via the citric acid cycle.

In this study adding more than the standard nitrogen fertilizer in the field during cultivation of *Narcissus* 'Carlton' did not lead to production of more galanthamine in the bulbs. The application of fertilizer did increase galanthamine compared to no fertilizer treatment, but adding more than the standard amount did not lead to further increases. Applying more nitrogen-containing fertilizer does not lead to the production of more alkaloids in all plants. Luanrata and Griffin[26] studied the effects of nutrients on a *Duboisia* hybrid (between *D. myoporoides* and *D. leichhardtii*) in hydroponic culture. Increased nitrogen application resulted in decreased hyoscyamine, scopolamine and other alkaloid yields. In a study on the growth and alkaloid content of *Tabernaemontana*, indole alkaloid accumulation increased in response to moderate fertilization, but was not further increased upon additional fertilization.[27]

Excessive fertilizer use is generally not recommended due to harmful effects on the environment, for example pollution of streams and lakes and increased greenhouse gas emissions.[28] The

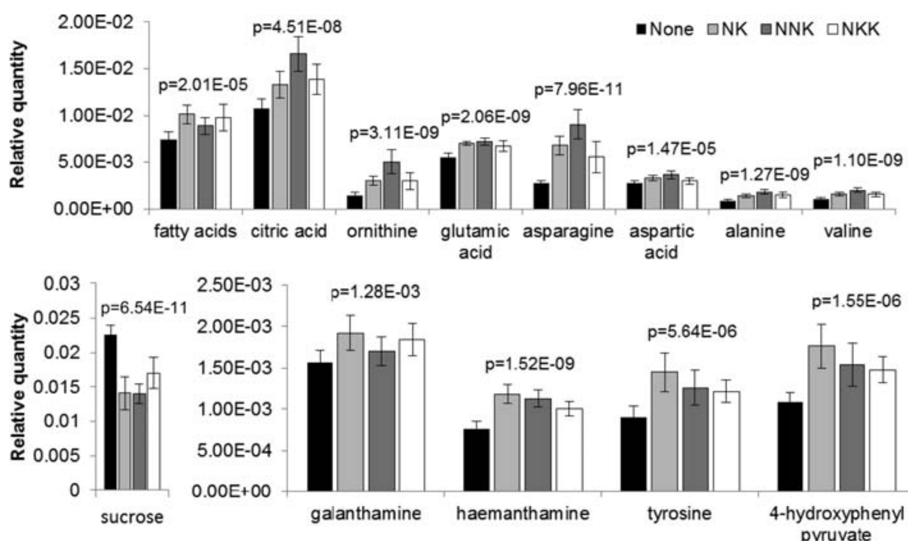

Figure 6. Relative quantification of compounds based on mean peak areas of associated signals. Chemical shifts of signals used were fatty acids: 1.31, citric acid: 2.56, ornithine: 1.76, glutamic acid: 2.16, asparagine: 2.95, aspartic acid: 2.63, alanine: 1.49, valine: 1.06, sucrose: 4.17, galanthamine: 6.16, hemeanthamine: 7.06, tyrosine: 7.18, 4-hydroxyphenylpuruvate: 7.13. The *p*-values of the ANOVA between the none, standard nitrogen and potassium (NK), double nitrogen and standard potassium (NNK) and standard nitrogen and double potassium (NKK) fertilizer treatment groups are shown on the graph.

use of excessive nitrogen fertilizer is also known to make bulbs more prone to infections with the fungal pathogen *Fusarium oxysporum* in *Narcissus*.[29,30] The standard levels of N and K as currently applied in The Netherlands is therefore sufficient for optimal galanthamine accumulation in the bulbs. This is favorable for farmers wishing to convert *Narcissus* from an ornamental to a pharmaceutical crop, as cultivation practices do not need to be changed and fertilizer cost will not increase.

This study illustrates how ^{1}H NMR-based metabolomic profiling can be used to assess the effects of cultivation practices on plant metabolism. This method allowed the quantification of the compound of interest, galanthamine, while also providing quantitative information on metabolites of a variety of chemical classes. Primary and secondary metabolites could be identified by comparison of analyzed samples to a library of NMR spectra measured under identical conditions. In one measurement a more global view of the bulb metabolism could be obtained. PCA showed differences between fertilizer treatments, which allowed for a better understanding of the effect of the treatments on alkaloid levels.

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Funding Sources

This project was funded in part by the Ministry of Agriculture of The Netherlands.

ACKNOWLEDGMENT

This study was done as part of the project "Inhoudstoffen bloembolgewassen, de basis voor een innovatieve keten" (project number 3030001607076). Dr. Young Hae Choi was supported by Nederlands Genomics Initiative (Horizon Project No.: 93519012).

ABBREVIATIONS USED

TMSP, trimethylsilylproprionic acid; PCA, Principal component analysis; HCA, hierarchical cluster analysis.

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