Chapter 6. Effects of fungicides on galanthamine and metabolite profiles in *Narcissus* bulbs

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Abstract

Large-scale plant cultivation usually involves the use of pesticides. Apart from eliminating the target organism, the external chemicals may affect the metabolism of the crop plant. This may have implications for plants cultivated for specific medicinal compounds. In this study the effects of diverse fungicides on the metabolism of *Narcissus pseudonarcissus* cv. Carlton bulbs were investigated. *Narcissus pseudonarcissus* cv. Carlton is being cultivated for the extraction of the alkaloid galanthamine. Fungicides typically used in *Narcissus* cultivation were applied in a field experiment. The aim was to determine whether fungicide applications changed the concentration of galanthamine in the bulbs. ^{1}H NMR spectroscopy allowed quantitative analysis of galanthamine and other metabolites in bulb extracts. Multivariate data analysis revealed changes in bulb metabolite patterns caused by fungicides. Bulbs treated before planting generally had higher levels of alkaloids, while foliar field applications caused lower alkaloid levels but altered carbohydrate metabolism. Within these groups, certain fungicide treatments caused changes in specific metabolites. This study shows that the fungicides used in *Narcissus* cultivation can cause a change in the metabolome still detectable in the bulbs after harvest. The standard cultivation practices in terms of fungicide treatment were found suitable for the production of *N. pseudonarcissus* cv. Carlton as raw material for galanthamine extraction. In the cultivation of medicinal plants for secondary metabolites the potential effect of pesticides and other agrochemicals should be taken into account.

Introduction

Conventional large-scale plant cultivation makes use of agrochemicals that allow high crop yields, efficient use of land and minimal loss to pests. Pesticide use is sometimes associated with problems of resistance of the target organism, persistence of the active compound or its metabolites in the environment, and unwanted effects on non-target organisms (Pimentel and Edwards, 1982; Urech et al., 1997).

The crop plants to which the pesticides are applied can also be considered non-target organisms (Mitra and Raghu, 1998). There are many reports on the effects of herbicides on crop metabolism (reviewed by Lydon and Duke (1989). It is not surprising that herbicides can have an effect on crop plants, as the biochemical target of the herbicide is often common to most plants. Effects of fungicides on plant metabolism have also been reported (Werbrouck and Debergh, 1996; Ypema and Gold, 1999; Gullino et al., 2000). The aforementioned studies mainly reported the effects of fungicides on crop physiology. Several studies also investigated the effect of such agrochemicals on plant secondary metabolites. In a review on the effect of pesticides on plant secondary metabolism, Lydon and Duke (1989) summarized some effects reported in the 1970s and 1980s. These included the capacity of certain fungicides to induce phenolic compounds and other phytoalexins, enhanced lignification in infected tissues, and accumulation of antifungal compounds such as α-linoleic acid. Garcia et al. (2000) reported increased phenolic compounds in tobacco leaves with low application rates of the biocide carbendazim, and decreased accumulation at higher application rates.

Whether in the cultivation of food, medicinal or other industrial crops, it is important to know how the application of pesticides will affect the crop. In the development of new agrochemicals/pesticides, the goal is to reduce adverse effects on the crop and other organisms. Apart from pesticide residues, these agrochemicals may affect the metabolism of the plant. This effect can be assessed on different levels, such as at the level of gene expression, enzyme activity or physiological responses. The end-products of all these metabolic processes and the determining factors of the quality of the crop are metabolites. Whether it is carbohydrates and vitamins in fruit and vegetables, aromatic compounds in herbs and spices, or other secondary metabolites in medicinal plants, these compounds may ultimately be affected by anything that alters the plant metabolism.

In this study, the effects of various fungicides on the metabolism of *Narcissus pseudonarcissus* L. (Amaryllidaceae) cv. Carlton bulbs were assessed on the level of metabolites. *Narcissus pseudonarcissus* is a widely cultivated ornamental crop well-

known for the many varieties of yellow trumpet-like flowers. The bulbs of many species have been found to contain alkaloids with a number of interesting bioactivities (Bastida et al., 2006). One of these, galanthamine, has been licensed as a drug against the symptoms of early Alzheimer's disease (Heinrich and Teoh, 2004). The bulbs of *N. pseudonarcissus* cv. Carlton are currently being cultivated as raw material for the production of galanthamine. There is a long tradition of *Narcissus* cultivation in the Netherlands, which has led to optimized cultivation practices with high yields of flowers and bulbs. Ornamental flower cultivation is characterized by high input of agrochemicals. Due to climatic and soil conditions in the bulb growing region, the major diseases (e.g. bulb rot, neck rot) of *N. pseudonarcissus* are caused by pathogenic fungi (Hanks and Carder, 2003). Thus cultivation of *N. pseudonarcissus* cv. Carlton typically involves treatment of the bulbs before planting in a water bath with warm or cold water to which fungicides are added. After planting, various pesticides are applied throughout the growing season. We wanted to know if the fungicide treatments have an effect on the plant metabolism, and specifically whether they affect the galanthamine content of the bulbs. A ^{1}H-NMR-based method was used as a method to quantify galanthamine, and at the same time determine whether changes occurred in the metabolite profile of the bulbs as a whole.

Materials and Methods

Plant production and pesticide treatment

Bulbs of *Narcissus pseudonarcissus* cv. Carlton were planted on November 13, 2008 in sandy soil in Lisse, The Netherlands. A plot was planted for each treatment, and a plot 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. All plots received the standard amount of nitrogen and potassium fertilizers (150 kg K_2O Patentkali, 30% K and 110 kg KS nitrogen, 15.5% N). A control plot was planted that received no treatment at all (treatment 1: control). Treatments 2-8 were treated before planting with fungicidal agents typically used in ornamental bulb cultivation. The bulbs were dipped in a tank of cold water for 15 minutes containing one or more agrochemicals as indicated in Table 1. Treatments 10-16 consisted of fungicide treatments in the field after the bulbs were planted. These treatments were sprayed at different growth stages as is typically done in ornamental bulb cultivation. Treatment 9 consisted of bulbs treated with a mixture of fungicides before planting and in the field. A summary of all the treatments is given in Table 2. Bulbs were harvested in July 2009, and were stored and dried at 20°C for two weeks. Bulbs were kept at 4 °C for about two weeks until further processing.

Table 1. Summary of fungicide treatments applied in water bath before planting.

no.	agrochemical applied	active substance	amount active substance in water bath (% w/v)
1	No pesticide	No pesticide	None
2	Formaldehyde	Formaldehyde	1.00 (v/v)
3	Luxan	Captan	0.273
4	Sportak	Prochloraz	0.09
5	Topsin	Thiophante methyl	0.25
6	Securo	Pyraclostrobin	0.15
		Folpet	0.45
7	Shirlan	Fluazinam	0.125
8	Mix 1: formaldehyde	Formaldehyde	1.00
	Luxan	Captan	0.273
	Sportak	Prochloraz	0.09
	Topsin M	Thiophanate methyl	0.25
9	Mix 2 : formaldehyde	Formaldehyde	1.00
	Luxan	Captan	0.273
	Topsin M	Thiophanate methyl	0.25
	Securo	Pyraclostrobin	0.15
		Folpet	0.45

Sample preparation

Nine healthy bulbs were selected from each treatment for sample preparation. For the control 18 bulbs were selected. The bulbs were washed to remove soil particles and the roots and basal plate was removed to aid grinding. Bulbs were frozen in liquid nitrogen before being individually ground to a fine powder in a Waring laboratory blender. Ground bulb material was freeze-dried for 7 days. Freeze-dried material was stored at -80 °C until extraction. The plant material was extracted using the standard metabolomics method developed in our group (Kim et al., 2010). Fifty mg of freeze-dried bulb material was transferred to 2 mL microtubes and 1.5 mL of a mixture of phosphate buffer and methanol-d_4 (1:1) containing 0.01% trimethylsilylpropionic acid sodium salt-d_4 (TMSP, w/w) was added. The samples were vortexed for 30 seconds, then extracted by ultrasonication for 30 minutes. After extraction samples were centrifuged for 5 minutes, and 1 mL of supernatant was collected. Aliquots of 800 µL were transferred to NMR tubes for measurement.

Table 2. Summary of fungicides applied to *Narcissus pseudonarcissus* plants in the field.

No.	Agrochemi-cal applied	Active substance	Amount ative substance (per ha)	Time of application (number of application)
9	Full mix:			
	Maneb	Maneb	1.60 kg	Before flowering (2)
	Kenbyo	Mancozeb	0.60 kg	During flowering (2)
		Kresoxim-	0.20 kg	After flowering (3)
	Shirlan	methyl	0.40 kg	
		Fluazinam	0.19 kg	
	Rudis	Prothioconazole		
10	Shirlan	Fluazinam	0.40 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
11	Allure	Prochloraz	0.21 kg	Before flowering (2)
		chlorthalonil	0.67 kg	During flowering (2)
				After flowering (3)
12	Maneb	Maneb	1.60 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
13	Kenbyo	Kresoxim-	0.20 kg	Before flowering (2)
		methyl	0.60 kg	During flowering (2)
		Mancozeb		After flowering (3)
14	Chlorthalonil	Chlorthalonil	1.00 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
15	Rudis	Prothioconazole	0.19 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
16	Field mix:			
	Maneb	Maneb	1.60 kg	Before flowering (2)
	Kenbyo	Mancozeb	0.60 kg	During flowering (2)
		Kresoxim-	0.20 kg	After flowering (3)
	Shirlan	methyl	0.40 kg	
		Fluazinam	0.19 kg	
	Rudis	Prothioconazole		

^{1}H NMR measurement

^{1}H NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 μs and an optimized relaxation delay (RD) of 5 s (Lubbe et al., 2010).

Data processing and analysis

^{1}H NMR spectra were Fourier transformed (LB = 0.3 Hz). Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to δ 0.0. For quantitative analysis of galanthamine, integration of 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 mg 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 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 (version 12.0 Umetrics, Umeå, Sweden) with Pareto scaling method. Analysis of variance (ANOVA) followed by Pairwise Multiple Comparison (Tukey Test) was performed to compare average galanthamine levels between treatments using SPSS (version 18, PASW Statistics, Chicago, IL, USA).

Results

Galanthamine quantification

Galanthamine concentration in the *Narcissus* bulbs was determined using a quantitative ^{1}H NMR method developed previously (Lubbe et al., 2010). The area under the doublet signal at δ 6.16 belonging to H-4a of galanthamine was used for quantitation. Figure 1 shows the average concentration of galanthamine per treatment. A one-way ANOVA revealed that a statistically significant difference in the average galanthamine concentrations between the groups ($F(15,132) = 21.54$, $p=0.000$). A Tukey *post-hoc* test revealed that two of the treatments before planting (treatments 3 and 4) had significantly lower levels of galanthamine (2.676 ± 0.463, $p=0.036$ and 2.248 ± 0.363 mg/g, $p=0.000$, respectively) than the control treatment (3.226 ± 0.221 mg/g). Field fungicide treatments that also had significantly lower levels of galanthamine were treatment 12 (1.591 ± 0.686 mg/g), treatment 13 (2.013 ± 0.603 mg/g, $p=0.000$), treatment 14 (1.618 ± 0.739 mg/g, $p=0.000$), treatment 15 (1.315 ± 0.660 mg/g, $p=0.00$) and treatment 16

(1.837 ± 0.683 mg/g, p=0.000). Although not statistically significant, the highest average galanthamine concentration was in treatment 9 (3.542 ± 0.199 mg/g) where mixtures of fungicides were applied before planting and in the field.

Metabolite identification

The ^{1}H NMR spectra were visually inspected and signals were assigned to various primary and secondary metabolites (Table 3, Figure 2). Identification of metabolites was done with the aid of two-dimensional NMR experiments (COSY, J-Resolved and HMBC), as well as comparison of signals with an in-house metabolite database and previously reported data (Verpoorte et al., 2007; Kim et al., 2010). In all samples, spectra were dominated by signals assigned to the disaccharide sucrose (Figure 2a).

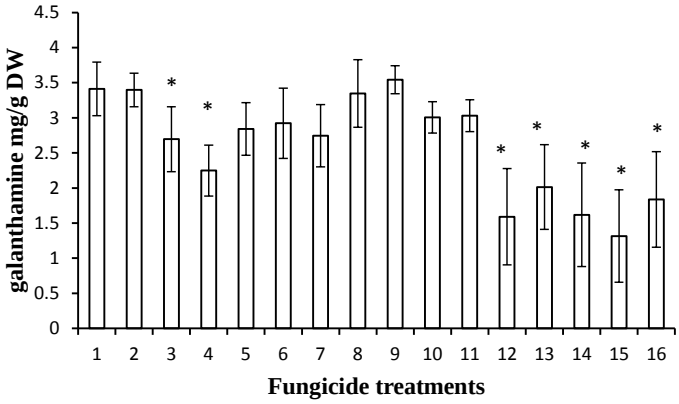

Figure 1. Average galanthamine content of *Narcissus pseudonarcissus* cv. Carlton bulbs treated with fungicides (mean ± SD, *P<0.05). Treatment 1: control (n=18), treatment 2-8: pre-planting fungicide treatments (n=9), treatment 9: pre-planting and in field fungicide treatment (n=9), treatment 10-16: in-field fungicide treatment (n=9). Treatment numbers are indicated in Table 1 and 2).

Glucose was identified by the doublet signals at δ 4.58 (J=7.9) and δ 5.19 (J=3.8) of the β- and α-anomeric protons, respectively. A doublet at δ 5.17 (J=3.8) was assigned to the H-1 of maltose with the help of COSY and HMBC experiments. Other minor sugars previously reported in *N. pseudonarcissus* bulbs (Ruamrungsri et al., 1999), raffinose and arabinose, were also identified. Signals belonging to galanthamine and other major alkaloids of *N. pseudonarcissus* cv. Carlton, as well as some biosynthetic precursors were identified in the region of δ 6-8 (Figure 2b). Galanthamine and haemanthamine signals were assigned as previously reported (Lubbe et al., 2010). The third most

abundant alkaloid narciclasine was identified with the help of two-dimensional NMR experiments and comparison of signals to the reports in the literature (Evidente, 1991). Two precursors in the alkaloid biosynthetic pathway in *Narcissus*, tyrosine and 4-hydroxyphenylpyruvate (4-HPP) were also identified (Bastida et al., 2006) (Figure 3).

Characteristic signals of various primary metabolites were seen at lower chemical shifts. This included the amino acids asparagine, aspartic acid, glutamic acid, ornithine, alanine, valine and threonine. The organic acids malic acid and citric acid were identified, as well as signals belonging to choline and ethanolamine. Characteristic triplet signals of fatty acid ω-protons were seen at δ 0.88, as well as the broad signal at δ 1.31 of their $(CH_2)_n$ chains. COSY and J-Resolved experiments also allowed identification of a triplet at δ 2.18 and multiplet at δ 1.56 of the -$CH_2\underline{CH_2}$COOH and -$\underline{CH_2}CH_2$COOH protons, respectively. In some samples a multiplet signal was seen at δ 5.40, partly overlapped with the large doublet of sucrose. This was assigned to the olifenic protons of unsaturated fatty acids, such as linoleic acid.

Table 3. ^{1}H Chemical Shifts (δ) and Coupling Constants (Hz) of *Narcissus pseudonarcissus* bulb metabolites in CH_3OH-d_4-KH_2PO_4 in D_2O at pH 6.0.

no.	Metabolite	Chemical shift (δ) and coupling constant (Hz)
1	Lycorenine	7.06 (s), 7.04 (s), 6.02(s), 5.73 (brs)
2	Phenylalanine	7.42-7.33 (m), 3.09 (dd) J=8.3, 14.8
3	Tyrosine	7.18 (d) J=8.4, 6.85 (d) J=8.4
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
5	*Cis*-aconitic acid	7.04 (s)
6	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)
7	Haemanthamine	7.06 (s), 6.71 (s), 6.51 (d) J=10.3, 6.36 (dd) J=10.3, 5.0, 5.97 (brs)
8	Narciclasine	6.22 (m), 6.09 (d) J=4.5, 6.73 (s), 4.39 (m), 4.32 (m)
9	Raffinose	5.55 (d) and 5.27 (d) J=3.8 (both COSY to 3.52)
10	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
11	Mannose	5.14 (d) J=1.5
12	Maltose	5.17 (d) J=3.8, 5.40 (d) J=3.9
13	Arabinose	5.23 (d) J=3.8
14	Rhamnose	5.11 (d) J=1.5, 4.87 (d) J=0.7, 1.28 (t) J=6.5
15	Glucose	4.58 (d) J=7.9 (β-anomer), 5.19 (d) J=3.8 (α-anomer), 3.20 (dd) J=8.8, 8.9
16	Choline	3.21 (s)
17	Ethanolamine	3.12 (t) J=5.3
18	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
19	Aspartic acid	2.82 (dd) 17.0, 8.5, 2.63 (dd) J=17.0, 9.5
20	Citric acid	2.71 (d) J=15.8, 2.56 (d) J=15.8
21	Malic acid	2.68 (dd) J=15.7, 3.4, 2.36 (dd) J=15.7, 10.4, 4.28 (dd) J=10.4, 3.2
22	Glutamic acid	2.39 (td) J=7.1, 2.5, 2.10-1.28 (m)
23	Ornithine	3.24 (t) J=8.0, 1.92 (m), 1.65-1.78 (m), 3.71 (t) J=5.8
24	Acetic acid	1.91 (s)
25	Alanine	1.49 (d) J=7.2
26	Valine	1.06 (d) J=7.0, 1.01 (d) J=7.0
27	Threonine	1.34 (d) J=6.6, 4.22 (m),
28	Fatty acids	1.31 (brs), 2.18 (t) J=7.4, 1.56 (m), 0.88 (t) J=7.4, 5.40 (m)

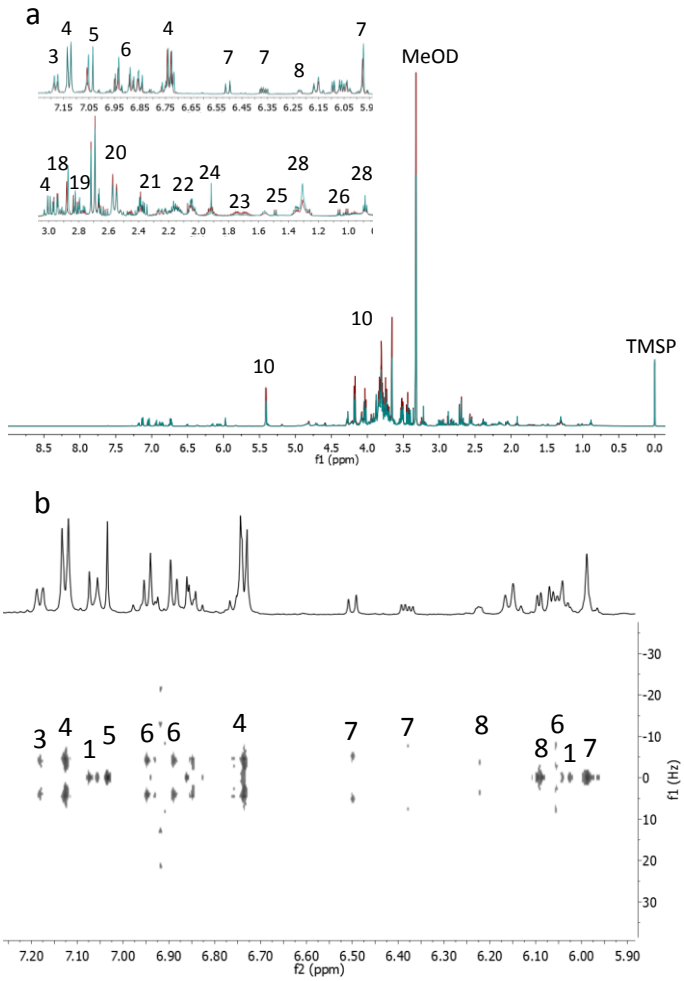

Figure 2. (a) Representative ^{1}H NMR spectra of *Narcissus pseudonarcissus* cv. Carlton bulbs of treatment 1 (control; red) and treatment 9 (fungicide mix 2; blue) extracted with phosphate buffer and methanol-d_4 (1:1), pH 6.0, showing metabolites occurring in different parts of the spectrum. 3: Tyrosine, 4: 4-Hydroxyphenylpyruvate, 5: *cis*-Aconitic acid, 6: Galanthamine, 7: Haemanthamine, 8: Narciclasine, 10: Sucrose, 18: Asparagine, 19: Aspartic acid, 20: Citric acid, 21: Malic acid, 22: Glutamic acid, 23: Ornithine, 24: Acetic acid, 25: Alanine, 26: Valine, 28: Fatty acids. (b) J-Resolved spectrum of the aromatic region of a representative *Narcissus pseudonarcissus* bulb extract. 1: Lycorenine, 3: Tyrosine, 4: 4-hydroxyphenylpyruvate, 5: *cis*-Aconitic acid, 6: Galanthamine, 7: Haemanthamine, 8: Narciclasine.

Multivariate data analysis

Multivariate data analysis was used to compare all the signals of all the ^{1}H NMR spectra. An unsupervised method, principal component analysis (PCA) was used to obtain an overview of the differences between bulb treatments in an unbiased way. PCA resulted in a model in which the first two principal components accounted for more than 70% of the variance in the dataset. The score scatter plot of the first two principal components is shown in Figure 4a. Each point in the score scatter plot represents one bulb sample. When the points in the score scatter plot are colored according to whether the treatment was applied before planting or after planting, a pattern emerges. Samples treated with fungicides before planting were roughly separated from those treated in the field along PC1, with the control samples clustered in the middle of the plot. Samples of treatment 9 grouped with the other samples that were treated with fungicides before planting.

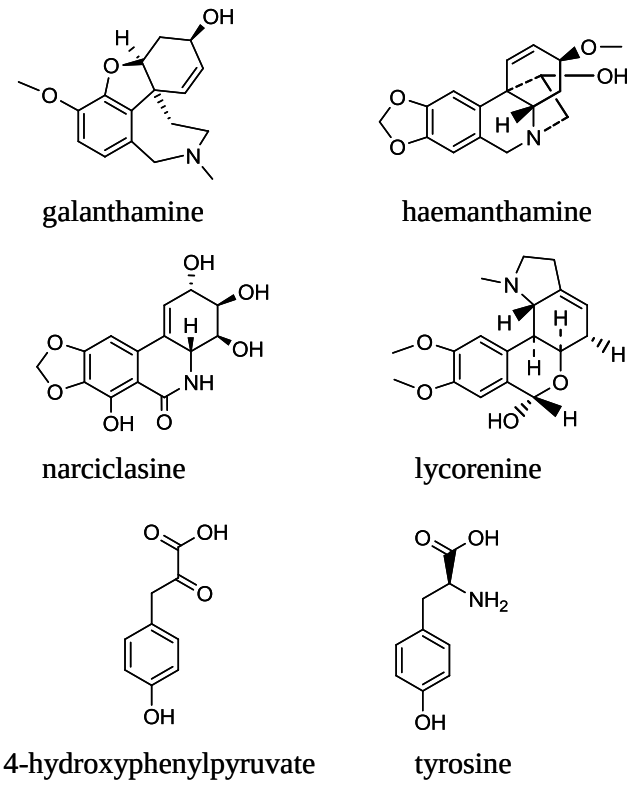

Figure 3. Chemical structures of some metabolites identified in *Narcissus pseudonarcissus* cv. Carlton bulbs.

A loading scatter plot of PC1 versus PC2 (Figure 4b) shows how different variables (buckets) contribute to the grouping of samples seen in the PCA score plot. Variables with a high positive loading on PC1 included buckets with signals assigned to sucrose, glucose and maltose (e.g. 3.80, 3.84, 3.64, 4.16, 3.44, 4.04, 3.68, 5.40, 3.36, 3.48 and 5.20). Variables with negative loadings on PC1 included buckets with signals assigned to alkaloids galanthamine (2.88) and haemanthamine (6.72), alkaloid precursors 4-HPP (3.00, 7.12) and tyrosine (6.88), amino acids aspartic acid (2.68) and glutamic acid (2.16, 2.40), organic acids citric (2.56, 2.72), malic (4.28, 2.36) and *cis*-aconitic acid (7.04) as well as fatty acids (1.36, 1.32, 0.88).

PCA was applied to those treatments which had a significantly lower concentration of galanthamine to see if any other changes in the metabolite profile might help explain the observed effect. PCA of controls and samples treated with captan (treatment 3) before planting gave a score scatter plot where the two groups were separated along PC1, with 42.7% of the variance accounted for (Figure 5a). From a loading column plot of PC1 the signals responsible for the groupings could be seen (Figure 6a). Signals with a negative loading included those of the alkaloids galanthamine, haemanthamine and narciclasine. Other signals with negative loadings on PC1 were those of tyrosine, aspartic acid, malic acid, citric acid, acetic acid and fatty acids.

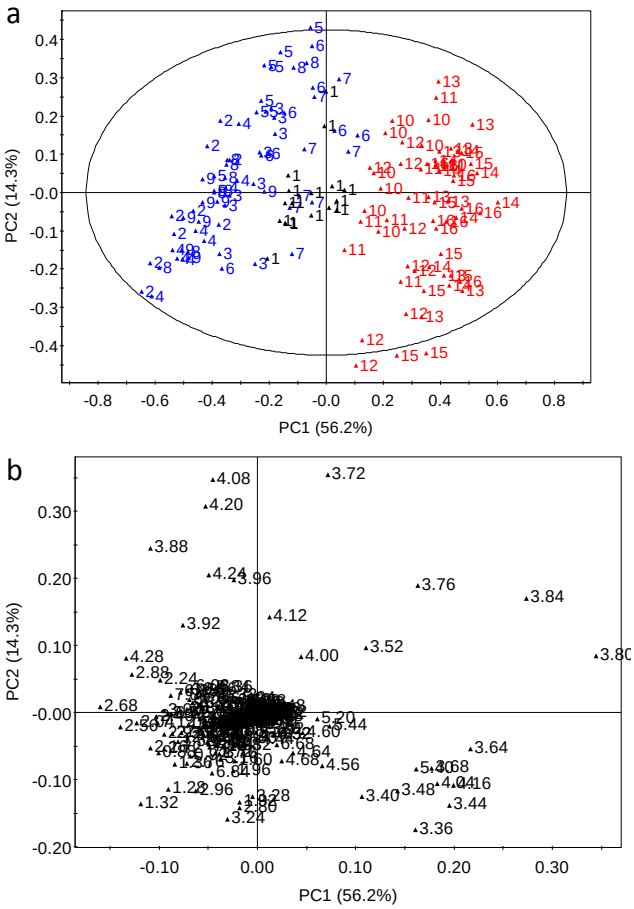

Figure 4. (a) Score scatter plot showing results of the PCA with all bulb samples. The numbers correspond to fungicide treatments as indicated in Table 1 and 2. (b) Loading scatter plot of PC1 versus PC2 of bulb samples treated with fungicides.

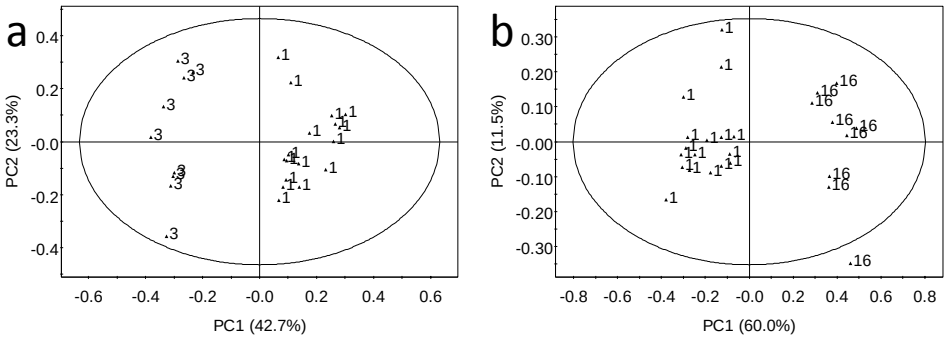

Figure 5. (a) Score scatter plot of PCA of control samples versus captan (treatment 3). (b) Score scatter plot of control samples versus field fungicide mix (treatment 16).

PCA of samples treated with prochloraz (treatment 4) before planting were separated from controls along PC1, with 38.4% of the variance explained. The treated samples were less tightly clustered and one outlier was seen outside the Hotelling's T range ellipse. Similar to the captan samples, signals responsible for the separation along PC1 were identified as the alkaloids (galanthamine, haemanthamine and narciclasine) as well as the alkaloid precursors tyrosine and 4-HPP. From the loading column plot further signals responsible for the separation in the score scatter plot were malic acid, citric acid, aspartic acid, acetic acid and ethanolamine. Two triplet signals (δ 1.19 J=7.0 and δ 1.12 J=7.4), which also contributed to the separation of the samples were observed in the prochloraz treatments but not in the controls. They were tentatively assigned to the methyl group of 1-*O*-ethyl-β-glucoside and terminal methyl group of a fatty acid, respectively. A double doublet signal at δ 5.50 (J=7.0, 3.5) also had a negative loading on PC1.

Treatments 12-16 were also submitted individually to PCA to compare their metabolite profiles with the control samples. In each case separation was seen between the treated samples and the controls along PC1 in a score scatter plot, with the first PC explaining between 40 and 60% of the variance. The variables responsible for the separation were similar in these field fungicide treatments. Figure 5b shows treatment 16 as an example. Signals common to all were those belonging to sucrose, the unsaturated fatty acid signal at 5.44, the dd at δ 5.50, fumaric acid, glucose and maltose (Figure 6b). In the maneb, rudis and field mix treatments formic acid contributed to the loading, and in chlorothalonil, rudis and field mix treatments raffinose had a positive loading.

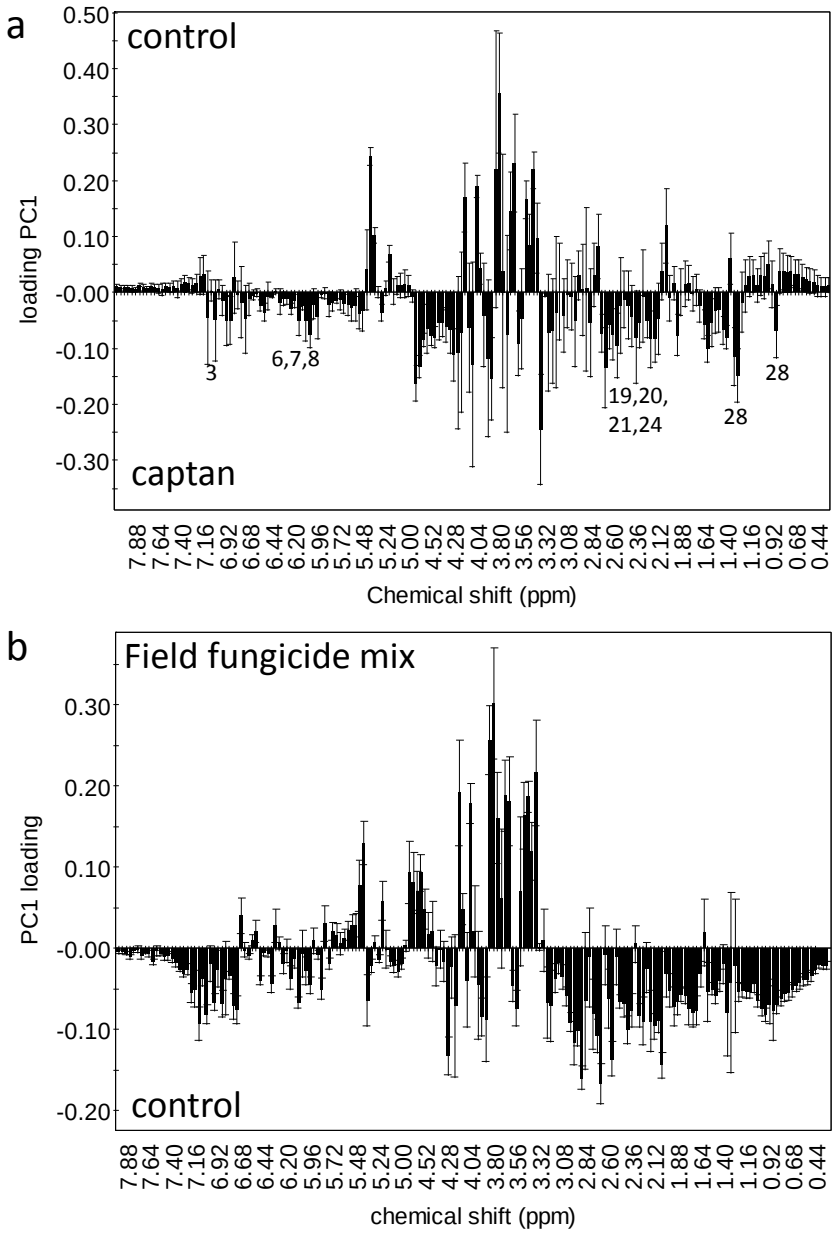

Figure 6. (a) Loading column plot of PCA of control versus captan, (b) loading column plot of PCA of control versus field fungicide mix.

Relative quantification of selected metabolites

The NMR bucket table was submitted to ANOVA, to compare the average signal intensities in each variable bucket and to confirm whether differences seen in the multivariate data analysis were statistically significant. The results confirmed what was found in the PCA, and some examples of buckets with significantly different average intensities are shown in Figure 6. The buckets containing tyrosine and haemanthamine signals (Figure 7a,b) for example, were higher in the before treatments. The treatments containing formaldehyde alone or in a mixture had the highest average levels. Signals belonging to sucrose were significantly higher in the samples treated after planting, as is shown in Figure 7c.

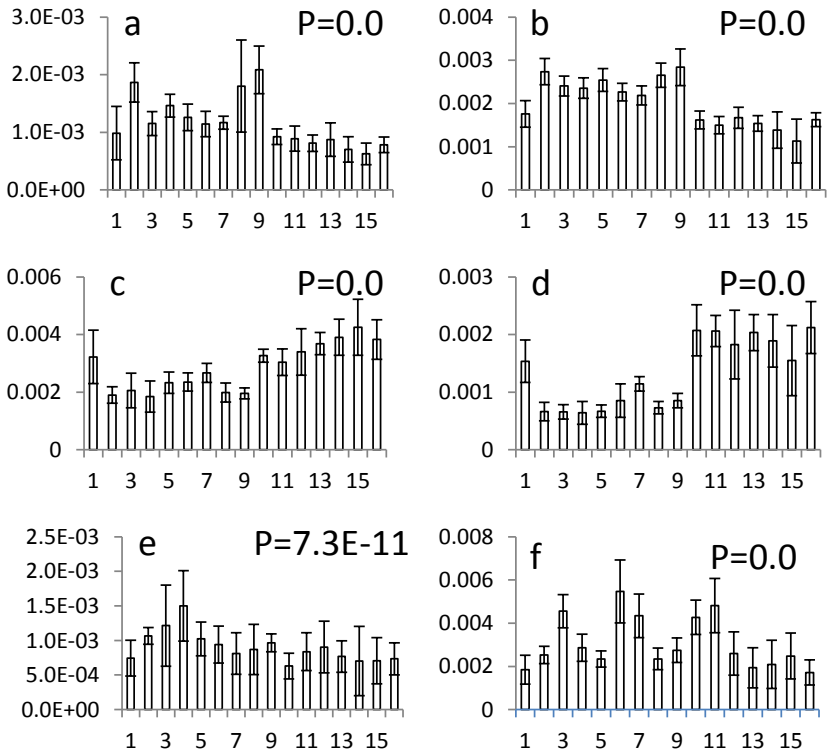

Figure 7. Relative quantification of selected compounds based on peak areas of associated signals (mean ± SD, n=18 for treatment 1, n=9 for other treatments). Chemical shifts were (a) 7.20: tyrosine, (b) 5.96: haemanthamine, (c) 5.44: sucrose, (d) 5.20: glucose, maltose, (e) 3.12: ethanolamine, (f) 2.60: citric acid. The P-values of the ANOVA between the different fungicide treatments are shown in the Figure.

The bucket of δ 5.20 contained signals of glucose and maltose. On closer inspection of this chemical shift region in all the samples, the relative ratio of the two doublet signals were seen to be different based on the timing of fungicide application. The maltose doublet signal intensity stayed constant between treatments. The glucose doublet signal at δ 5.19 was approximately double the size of the maltose doublet signal at δ 5.17 in the control. In samples treated before planting, the doublet signals were about equally intense. In contrast, samples treated after planting also had more intense glucose doublet signals, but with more than double the intensity of the doublet at δ 5.17. This is shown in Figure 8, where these signals of representative samples from the different times of application are compared.

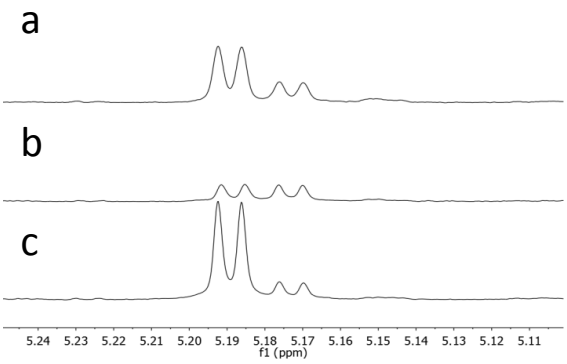

Figure 8. The doublet signals of glucose (δ 5.19) and maltose (δ 5.17) in a representative ^{1}H NMR spectrum of (a) control bulbs (treatment 1), (b) bulb treated with captan (treatment 3), (c) bulb treated with fluazinam after planting (treatment 10).

Discussion

In conventional agricultural systems, applied fungicides may affect the metabolism of the crop plant. This can have important implications for plants cultivated for the production of medicinal compounds. ^{1}H NMR was used to analyze the fungicide-treated *N. pseudonarcissus* cv. Carlton bulbs, which allowed the detection and quantitative analysis of major primary and secondary metabolites. A targeted analysis of galanthamine in the bulb samples revealed differences in the concentration between certain treatments. The average galanthamine concentration was as low as half that of the control bulbs in some treatments (Figure 1). Analysis of the entire ^{1}H NMR spectrum by PCA revealed grouping based on the comparison of all the variables between samples. The PCA score plot revealed that the time of application of fungicides was an important factor in explaining the variance in the dataset. The bulbs treated with

fungicides before planting generally had more intense signals in the aromatic region of the ^{1}H NMR spectrum. Galanthamine, haemanthamine, narciclasine and the alkaloid precursors tyrosine and 4-hydroxyphenylpyruvate were important for the discrimination. While these signals were also present in the other samples, they were less intense in the control samples and those treated in the field. Similarly, major sugar signals were seen in all the samples, but those belonging to sucrose were higher in bulbs treated in the field. Changes in carbohydrate metabolism were also seen by the altered ratios of glucose relative to maltose in different treatments compared to the control.

The samples treated with the sterol biosynthesis inhibitor (SBI) prochloraz (treatment 4), had a lower average galanthamine concentration and higher ethanolamine levels than the control. Increased ethanolamine suggests an effect on the metabolism of cell membrane components (Rontein et al., 2003). Two metabolite signals (1-*O*-ethyl-β-glucoside and fatty acid) were also seen only in this fungicide treatment. The contact fungicide captan (treatment 3) works on multiple biochemical targets to affect fungal pathogens. Bulbs treated with this fungicide also had a lower average galanthamine concentration. This is in spite of having more intense signals in the alkaloid and aromatic compound region, as for the other samples treated before planting. A shift in the bulb metabolism was caused by this fungicide treatment, as seen by altered levels of amino acids and organic acids, in particular citric acid.

The field fungicide treatments that had lower galanthamine concentrations were characterized by increased unsaturated fatty acids, as well as increases in certain organic acids. Changes in sugars were also seen as compared to the controls. These differences as well as the lower galanthamine levels may all be a reflection of the effect of the fungicides on metabolism of the photosynthetic tissue in the field. Bulbs are storage organs and much of the products of photosynthesis and other metabolites are transported down to them when the aboveground parts senesce (Hanks, 2002). The effect of foliar fungicide treatments can still be seen in the metabolite profiles of the bulbs after leaf senescence.

A study by Kucht et al. (2004) describes a decrease of alkaloids as a result of fungicide application in *Ipomoea*. This was ascribed to the inhibitory effect of the fungicide on an endophytic fungus, which was hypothesized to be involved in alkaloids' production. One could speculate that the foliar fungicide applications altered the amount of galanthamine and other alkaloids as a result of their effects on an associated fungus. *Narcissus* roots are known to be associated with mycorrhizal fungi (Chilvers and Daft, 1981), however the occurrence of any leaf endophytes is not known. Changes in metabolite patterns in the fungicide-dipped bulbs may also be due to a direct or indirect (via the plant) effect on symbiotic microorganisms (Diedhiou et al., 2004). Studies on

the effects of different fungicides on mycorrhizal symbioses showed mixed results, depending on the agent applied (Diedhiou et al., 2004). A study on the effects of two different SBIs on carrot roots showed one to alter root sterol composition and have a toxic effect on the symbiosis, while the other had minimal impact on plant sterols and fungal colonization (Campagnac et al., 2008). The authors emphasized the importance of studying the impact of fungicides on the functioning of symbiotic interactions in crops. The decreased galanthamine in plants with certain fungicide applications as compared to the control could suggest such indirect effects. However, such interactions are difficult to demonstrate clearly in a field study.

The approach used in this study was able to address the question of whether fungicide applications in *N. pseudonarcissus* cv. Carlton cultivation affect the galanthamine concentration in the bulbs. For most of the treatments the galanthamine concentrations were similar. The treatments with lower levels were all with individual fungicides applied either before or after planting. In the large-scale cultivation of *N. pseudonarcissus* cv. Carlton mixtures of fungicides are typically used. In this study the treatments that received a mixture before planting (treatment 8), and a mixture before planting as well as in the field (treatment 9) in fact had the highest average galanthamine concentrations. This was in spite of the mixtures containing one or more of the fungicides that had lower galanthamine in the individual treatments. The standard cultivation practices for *N. pseudonarcissus* cv. Carlton in terms of fungicide treatment thus seem suitable for the cultivation of the plant as raw material for galanthamine extraction, at least in terms of its effect on the compound of interest. In the cultivation of medicinal plants for secondary metabolites the potential effect of pesticides and other agrochemicals should be taken into account.