Factors affecting galanthamine production in Narcissus

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Chapter VIII

Metabolic Analysis of Basal Rot Infected *Narcissus* Bulbs and Metabolic Variations due to Infection point

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Abstract:

Narcissus bulbs are mostly grown as a crop for both bulb and flower production in several countries. Without proper care, its production is greatly affected by a fungal disease called basal rot. It is the most important disease of this crop, which is caused by *Fusarium oxysporum* Schl. The pathogen causes rotting of bulbs in the soil during the growth season and also when stored after harvesting. Phytosanitary failure is a major problem for the bulbs which are destined for export. This causes considerable financial losses. Among the fungal infections, another disease (neck rot) is quite like the basal rot at the later stages of infection. According to previous studies, its symptoms are mostly associated with *Fusarium oxysporum, Botrytis narcissicola* and *Penicillium hirsutum*. All these pathogens were found in the bulb stocks which were infected by neck rot. Thus, making it quite difficult to determine which one of these three is the causal agent for this disease. Though later, after multiple studies, it has been narrowed down to only *Fusarium oxysporum*.

The aim of this study was to compare the levels of galanthamine and other alkaloids in the infected bulbs and the control and to possibly identify biomarkers for each rot. A non-targeted metabolomics analysis was performed. Different methods could be used for the determination of metabolites in plant samples, but ^{1}H-NMR spectroscopy provides the best overview of quantitative differences in metabolites. Due to this reason ^{1}H-NMR spectroscopy was used in this study. The results showed that both types of rot have the same causal agent, but they affect the bulbs differently depending on the site of the attack in *Narcissus* plants. Bulb mass was significantly lower due to basal rot and affected plants showed stilted growth. Galanthamine levels in basal rot bulbs showed a significant

increase if compared to the controls while in the case of neck rot the difference was not very prominent between control and infected bulbs.

Keywords: *Narcissus*, fungal infection, basal rot, neck rot, galanthamine, NMR spectroscopy, metabolomics.

Introduction

Fusarium species are pathogenic fungi which are found worldwide in human and animal food such as cereals. These microorganisms cause great economic problems for the agricultural sector [1, 2]. In the European Union, it is estimated that about 20% of the total grown crops are affected by *Fusarium* [3]. Though most species are found in the field, some species are capable of growing in stored grains especially in the presence of freely available water (available water close to 0.99 g/m^3) and low temperature [4]. Generally, these species are plant pathogens but recent studies have reported that *Fusarium* is an emerging human pathogen in immune compromised patients [5].

There are speculations that pathogenic forms may have evolved from originally nonpathogenic strains because there are both pathogenic (both plant and human) and nonpathogenic strains [6]. Among all the species, *Fusarium oxysporum* is the most common species which affects a wide variety of economically important crops by causing vascular wilt disease. Widely cultivated plant taxa are usually not the hosts to pathogenic forms of *Fusarium oxysporum* except the grasses and most tree crops. According to their host range, there are 120 different forms which can be identified among *Fusarium oxysporum* isolates [5]. *Fusarium oxysporum* behaves as a classical soil borne pathogen because propagates of this fungus can survive in the soil for extended periods of time in the absence of the host. After germination and penetration in the host roots, however, the fungus enters the plant vascular system and subsequently uses the xylem vessels as avenues to rapidly colonize the host, thereby provoking the characteristic wilt symptoms. Successful infection includes a number of processes such as early plant-host signaling, root surface attachment, degradation of physical host barriers, resistance to host antifungal compounds and production of phytotoxins [7, 8].

Narcissus flowers are a big product in Dutch horticulture industry. The bulbs are produced for both bulb sale and flower production, but the production is affected greatly by basal rot. Basal rot, the most important disease of the *Narcissus* crop, is caused by *Fusarium oxysporum* Schl. formae speciales (f.sp.) narcissi Snyder & Hansen [9, 10]. The pathogen causes rotting of bulbs in the soil as well as in the storage. It is also the major cause of phytosanitary failure in bulbs destined for export. *Fusarium oxysporum* f.sp.

narcissi exists in the soil as chlamydospores. These chlamydospores, in favorable conditions, can survive for up to 20 years. However, for practical reasons, growers may be able to only achieve a 5-7 year cycle of crop rotations. Spores carried on the outside of diseased and healthy bulbs together with chlamydospores in the soil are the main reservoir for infection [11]. The initial symptoms are the stilted growth and the premature dying of the foliage with no flowers at all or very limited number of flowers. This ultimately leads to soft bulbs which turned into mummified bulbs after full infection with rot spreading upwards from the base plate. At this point recovery of the bulb usually becomes impossible. The bulbs can also be infected in storage if infected bulbs are present in the bulk. There are different methods to control this disease which includes chemical as well as biological control such as fluorescent Pseudomonas and non-pathogenic Fusarium. Chemical methods include different fungicide sprays, while presently the most common method of control is treating bulbs with hot water (44.4 °C for 3 hours) containing formaldehyde and thiabendazole hypochlorite for disinfection before planting in the field. The use of formaldehyde is likely to be discontinued because it is environmentally undesirable, moreover resistance to thiabendazole hypochlorite has been reported [12].

Neck rot is another fungal disease of *Narcissus* which was first reported in the early 1930s. It starts from the neck of the bulb as the name suggests and from the dead bases of the leaves in the neck of the bulb. It spread down towards the base of the bulb which causes the lack of shoot emergence. It can attack more cultivars than base rot [13]. Neck rot in the later stages is quite like the basal rot and its symptoms in post-harvest *Narcissus* bulbs in the 1970s and 1990s were associated with *Fusarium oxysporum, Botrytis narcissicola* and *Penicillium hirsutum*. Their frequencies in the infected bulbs and the incidence of neck rot were similar in the studies in these two decades. *Fusarium oxysporum, Botrytis narcissicola and Penicillium hirsutum* were isolated from neck rot infected stocks and from the necks of the bulbs. In the 1990s, *Fusarium oxysporum* was found in 51% of stocks of bulbs which were infected with rots. *Botrytis narcissicola* and *Penicillium hirsutum* were found in 76% and 92% of the stocks, respectively, and were isolated from rots of the neck, rots extending to the middle of the bulb and from completely rotted bulbs [14]. A survey of crop husbandry practices in the 1990s revealed

that in fact only the presence of *Fusarium oxysporum* was related to the stock problem. *Botrytis narcissicola* was isolated from fewer bulbs when grown in the fields that were planted previously with cereals or for bulb crops treated with foliar sprays of dicarboximide fungicides and pre-store dip treatments of MBC (benzimidazole) or formaldehyde. There were fewer bulbs infected with *Penicillium hirsutum* when bulbs had been planted at depths of 15 cm or less, following dicarboximide and MBC foliar treatments but more bulbs had *Penicillium hirsutum* infections after bruising. Eventually, in a project on *Narcissus* neck rot it was found that although *Penicillium hirsutum* and *Botrytis narcissicola* were isolated from the neck rot infected bulbs, there is clear evidence that *Fusarium oxysporum* is the sole responsible organism for this type of rot [15]. It is evident from the reports that *Fusarium oxysporum* is the causal agent for both type of rots but only the site of attack is different. So, it is important to find metabolomic changes as markers for both rots to scan for possible rot in batches of bulbs.

The aim of this study was to determine the metabolic changes in *N. pseudonarcissus* bulbs infected with basal bulb rot disease at different stages using a quantitative ^{1}H-NMR method developed previously [19]. The NMR spectra were analyzed by PCA to observe effects on overall metabolite profiles of the Narcissus plants. Moreover, differences between neck rot and basal rot diseases were compared to find markers for these diseases.

Nuclear magnetic resonance (NMR)-based metabolic profiling is a good approach to assess the effects of cultivation practices on plant metabolism as a whole. NMR spectra can give useful qualitative and quantitative information about a sample in a single measurement, without being compound class selective [16]. After acquiring NMR spectra, they are processed to extract the data. The most common way is through “binning” or “bucketing”, in which spectra are split into discrete regions and integrated [17]. Multivariate data analysis methods such as principal component analysis (PCA) are often used to analyze the data. PCA compares all data “buckets” (variables) between samples simultaneously. This enables clustering of samples to reduce the dimensionality of the dataset and reduce the number of variables needed to describe it [18]. Through further analysis, the spectral areas responsible for grouping in the samples can be investigated, and responsible compounds can be identified [16].

Material and Methods:

Chemicals and solvents:

For the NMR analysis the following chemicals were used: methanol-d_4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA), and phosphate (KH_2PO_4) buffer (pH 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylproprionic acid sodium salt-d_4 (TMSP, w/w) as an internal standard for quantitation and calibration of the chemical shifts.

Fusarium culturing and plant treatment:

Fusarium was obtained from the department of molecular microbiology on a single petri dish. This was sub-cultured and multiplied on solid potato dextrose agar (PDA) at room temperature (24 °C). Sterilized water was used to collect the conidia by immersion from the culture while sticky spores were removed with the help of sterilized toothbrush from agar media. This suspension of the conidia was then collected with the sterilized pipettes and filtered through a double layer of Mira-cloth. Concentrations were checked and adjusted to 10^6 spores/ml by using sterilized distilled water. After obtaining sufficient amount of *Fusarium,* it was mixed with two parts of soil and one-part sand in a mixer. This mixture of soil, sand and fungi were then filled in the pots for bulb planting. Bulbs were planted in the infected soil under the greenhouse conditions (temperature 25 °C, humidity 50-60%, 16 hours light and 8 hours dark) and appropriately watered every third day till the final harvest. Harvesting of the bulbs was started after three weeks from the date of planting. In each harvest, seven (7) bulbs were harvested while control bulbs were harvested separately at the same time point. All the harvestings were carried out after every three days from the previous harvest over a time period of three weeks. After harvesting, bulbs were rinsed to remove any remaining soil particles. All the roots were also removed from the control and infected bulbs. These bulbs were then chopped, ground to a powder and freeze dried. The bulbs with the neck rot (fully rotten) disease, kindly provided by Holland Biodiversity BV, were subject to the same procedure.

Metabolite extraction and NMR spectra measurements

Freeze-dried plant material (50 mg) was transferred to a 2-mL microtube and vortexed at room temperature for 1 minute with 1.5 mL of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethyl-silyl-propionic acid sodium salt (w/w) (TMSP) and methanol-*d4* (1:1). The sample was then sonicated for 20 minutes and centrifuged at 13,000 rpm for 10 minutes. An aliquot of 800 µL of the supernatant was used for NMR analysis. ^{1}H-NMR, including 2D-J-resolved spectra, were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). ^{1}H–^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600-MHz Bruker DMX-600 spectrometer (Bruker). All the NMR parameters were the same to those of the previous studies [19-22].

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 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 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 and Discussion

Bulbs of *Narcissus pseudonarcissus* were harvested during the experiment at different time points after three weeks of transplanting. Early symptoms of basal rot which were visibilly observed included stunted leaf growth and early senescence in many cases. There were minimum to low number of flowers in most of the cases. To check the effect of basal rot on total yield (**Figure 8.1)**, fresh bulbs were weighed soon after harvesting from the pots. From the harvested bulbs It was evident that most of the infected bulbs had lost the roots depending on the severity of infection. Also from the visual observations and weighed data, it was obvious that basal rot had negatively affected the bulbs. To verify these observations, data of the weighed fresh bulbs was subjected to two-way ANOVA with health and severity of infection as two main factors. Health main factor had two treatments (healthy/control, diseased/basal rot) while severity of infection had seven treatments (days of harvest (3, 6, 9, 12, 15, 18 and 21 days) from three weeks of transplanting). From the statistical analysis it was clear that both main effects (health and infection severity) as well as their interaction had a significant effect on the fresh bulb yield at the .05 significance level. The main effect of health yielded an F ratio of $F(1, 84) = 6.89248$, $p = .00039$ which showed a significant effect. While the main effect of disease severity yielded an F ratio of $F(6, 84) = 7.59497$, $p = .00086$ which showed a significant effect. At the same time, their interaction (type of cut × time after cut) yielded an F ratio of $F(6, 84) = 8.02729$, $p = .00979$, indicated that difference in bulb yield were present when bulbs were infected and severity of the disease was varied. Post-hoc (Dunnett's test) revealed that highly severe disease ($M = 26 \pm SEM = 2.5$) conditions (days 18 and days 21) had the highly significant effect when compared with control sample ($M = 58.7 \pm SEM = 3.48$) at the same time point. While medium infected ($M = 38.4 \pm SEM = 1.85$) conditions (days 12 and days 15) had a significant effect when compared with the control ($M = 56 \pm SEM = 2.8$) sample at the same time point. It was evident from the statistical results that infection severity negatively affected the bulb weight and fresh bulbs lost almost half their weight due to the infection and its progression.

Bulbs of *Narcissus* contain a wide range of metabolites. To observe the changes in these metabolites, bulbs were extracted with the above-mentioned method and subjected to ^{1}H-NMR analysis. The ^{1}H-NMR spectra (**Figure 8.2**) of control and basal rot samples were

visually inspected and compared. Signals were assigned to various primary and secondary metabolites after comparing with previous studies and the in-house spectral library. Major signals in the ^{1}H-NMR spectra were assigned to primary metabolites such as sucrose, citric acid, fatty acids and various amino acids and alkaloids (**Table 8.1**). Signals belonging to galanthamine and haemanthamine, the two most abundant alkaloids in this cultivar of *N. pseudonarcissus* were also identified. Signal assignments in this NMR solvent matched those previously reported [19, 20, 23] and were confirmed using an in-house-built database of ^{1}H-NMR spectra of metabolites from plants.

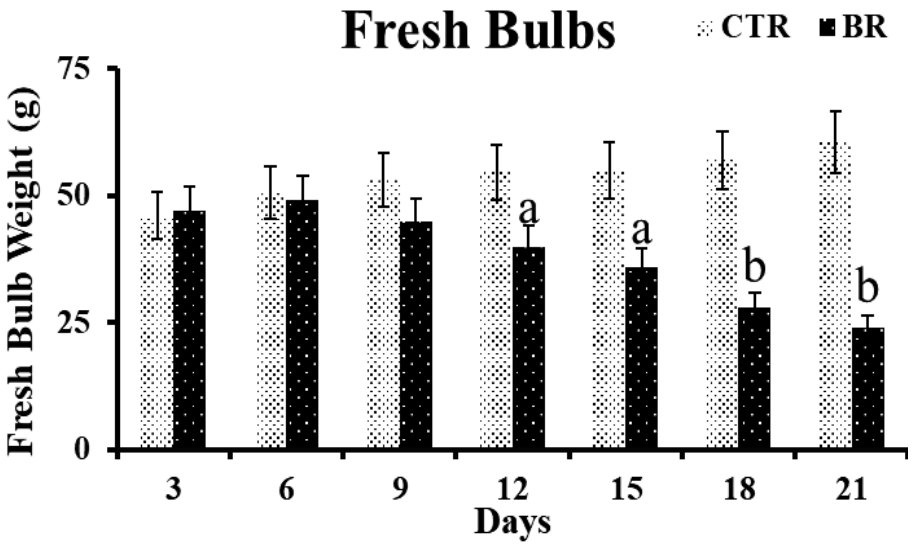

Figure 8.1. Fresh bulb weight of the basal rot infected bulbs compared with control at different stages of plant growth. Harvesting was done every after 3 days at the same time for control (CTR) and basal rot (BR) infected samples. Analysis of variance with Dunnett's test was used for comparison of galanthamine between healthy and infected samples at the same time of harvesting (7 biological replicates significance was at (a) $P < .05$ and (b) $P < .01$, error bars represent standard error (SEM).

Principal component analysis (PCA) was used to reduce the complexity of the ^{1}H-NMR data and to highlight the compounds which caused the maximum variation between the samples. In the score plot of PC1 and PC2 (**Figure 8.3A)**, control samples were clearly separated from the basal rot infected samples. Although there was clear separation between the control and the infected samples, there is a lot of overlap between the infected samples when severity of infection or time after infection are considered.

Table 8.1. ^{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)
Haemanthamine	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	5.19 (d) J=3.8, 4.58 (d) J=7.9, 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, 3.83, 2.81 (dd) J=17.0, 8.23
Aspartic acid	2.82 (dd) J=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=, 3.71 (t) J=5.8, 1.92 (m), 1.65-1.78 (m),
Alanine	1.49 (d) J=7.2
Valine	1.06 (d) J=7.04, 1.01 (d) J=7.04
Fatty acids	1.31 (brs), 0.89 (t) J=7.1,

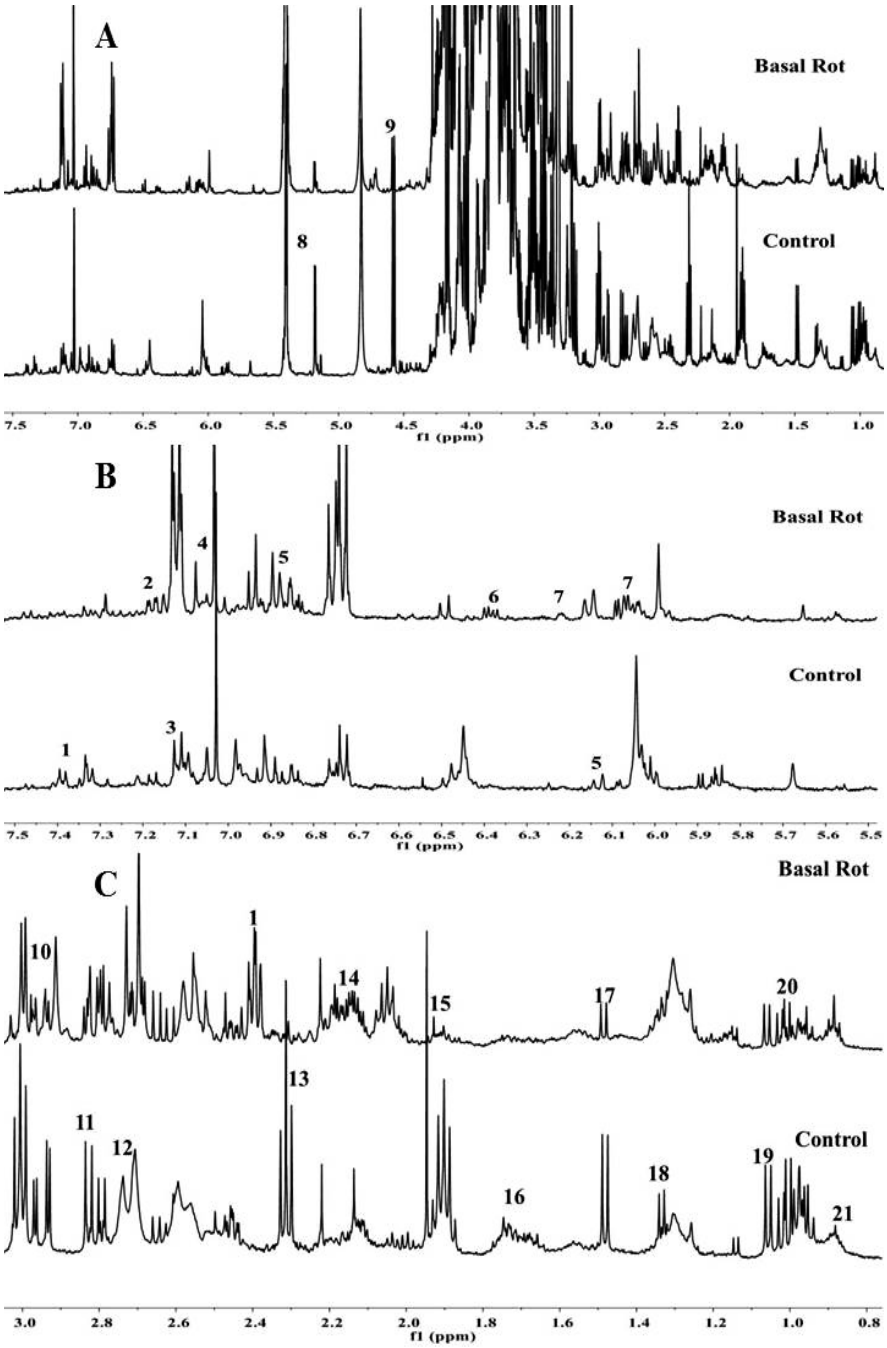

Figure 8.2. A) ^{1}H-NMR spectra (500 MHz) of *Narcissus pseudonarcissus* control bulbs and basal rot infected bulbs extracted with deuterated aqueous phosphate buffer and methanol-*d4* (1:1), range of δ 7.5-0.8 ppm; B) range of δ 7.5-5.5 ppm; C) range of δ 3.0-

0.8. Compounds: 1 :phenylalanine, 2: tyrosine, 3: 4-hydroxyphenylpyruvate, 4: *cis*-aconitic acid, 5: galanthamine, 6: haemanthamine, 7: narciclasine, 8: sucrose, 9: glucose, 10: asparagine, 11: aspartic acid, 12: citric acid, 13: malic acid, 14: glutamic acid, 15: acetic acid, 16: ornithine, 17: alanine, 18: threonine, 19: valine, 20: isoleucine, 21: fatty acids.

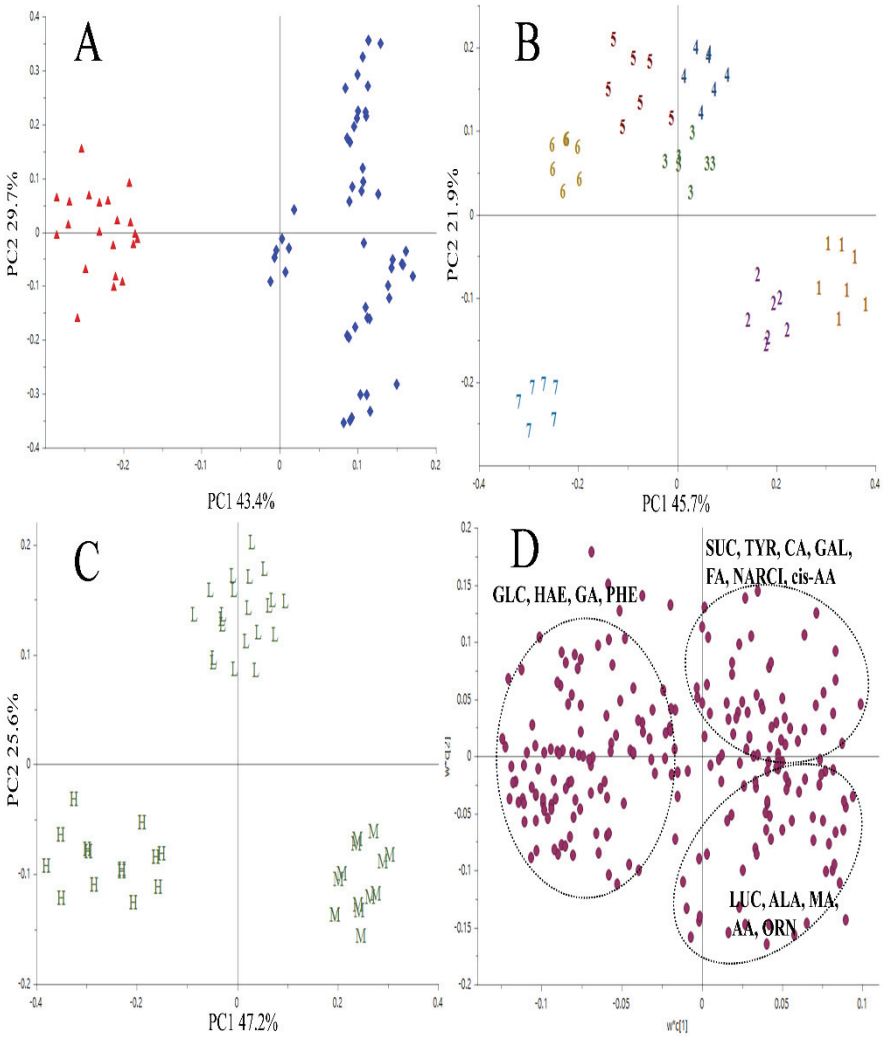

Figure 8.3. A) Score plot of PCA (PC1 vs PC2) of ^{1}H-NMR spectra of extracts of *Narcissus pseudonarcissus* bulbs (●) control bulb samples (○) all samples infected with basal rot, ignoring the severity of infection.

B) Score plot of the OPLS-DA of basal rot infect bulbs divided into seven classes based on time point of harvesting.

C) Score plot OPLS-DA of infected bulbs with 3 classes based on severity of infection (L) samples with low infection; (M) samples with medium infection; (H) samples with high infection.

D) Loadings-plot of bulbs where PC1 represent control to infection and PC2 represents time after infection and signals are assigned to Galanthamine (GAL), Haemanthamine (HAE), Narciclasine (NARCI), Phenylalanine (PHE), Tyrosine (TYR), Sucrose (SUC), Glucose (GLC), Leucine (LEU), Alanine (ALA), glutamic acid (GA), cis-aconitic acid (cis-AA), malic acid (MA), Citric Acid (CA) and Fatty acid (FA). PLS-DA models were validated by using permutation test (R^2 = 0.81, Q^2 = 0.69 with 120 permutations and 5 components)

To identify samples based on time after infection, the supervised method orthogonal partial least squares Discriminant Analysis (OPLS-DA) was used. An OPLS-DA scatter plot of PC1 vs. PC2 (**Figure 8.3**B) shows a clear separation between samples based on time after infection, i.e. related to the increasing severity of the infection. In this analysis only, infected samples were used. From the corresponding loadings plot, different spectral areas contributing to the grouping of samples seen in the PCA score plot were found. The discriminating signals belong to organic acids and amino acids which are high in the early stages of infection. The signals associated with the later stages of rot infection were mostly above δ 5.4 and associated with sugars and phenolics. At visual inspection and identification, 4-hydroxyphenylpyruvate showed a marked increase and can be considered as a biomarker for the identification of basal rot infected bulbs. 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. To determine the difference between different levels of infection, a supervised analysis method (OPLS-DA) was used where samples were divided into three different classes based upon the level of infection i.e. low, medium and high. Results (**Figure 8.3**C) clearly indicate that there are differences between the classes depending upon the level of infection as classes were clearly separated.

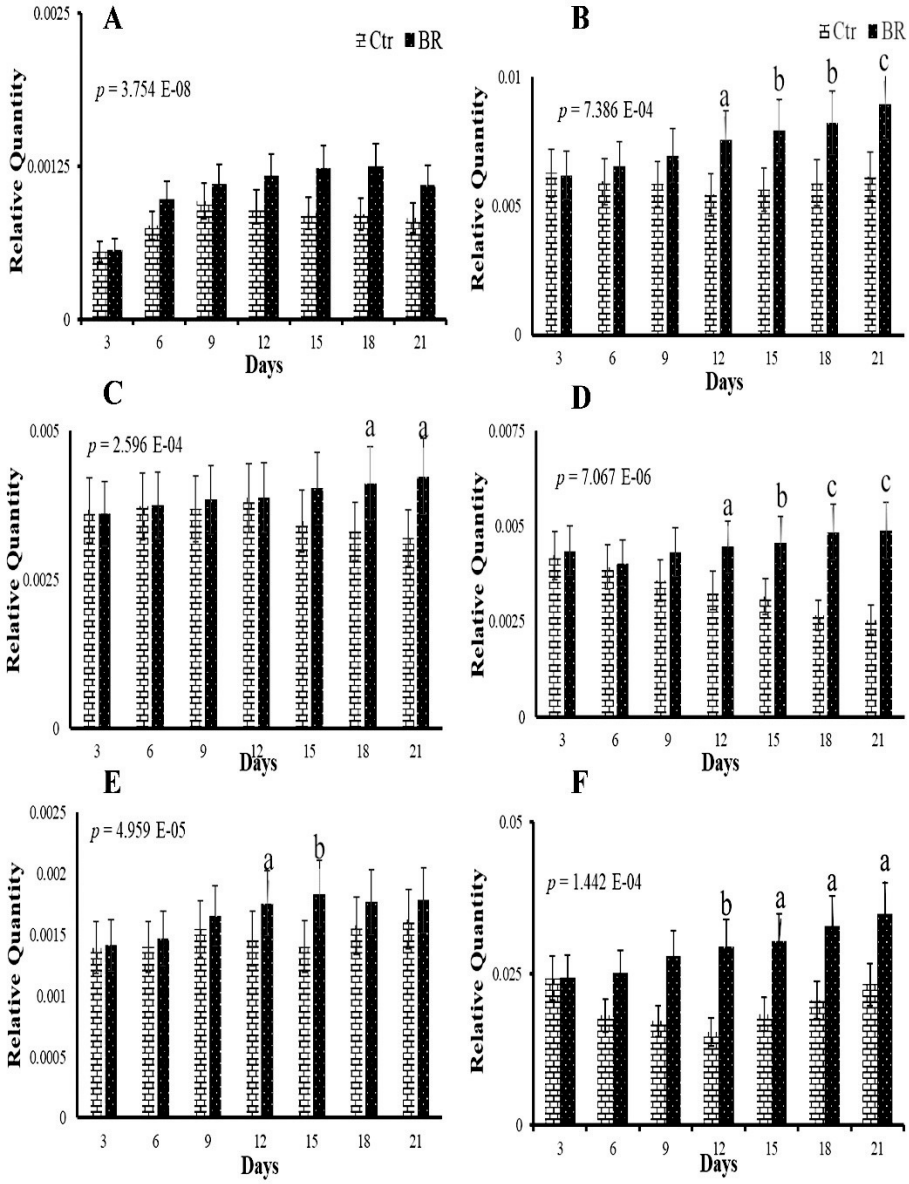

Figure 8.4. Relative molar quantification of compounds *Narcissus pseudonarcissus* controls (Ctr) and basal rot bulbs (BR) calculated from areas of ^{1}H-NMR signals (500 MHz).Chemical shifts of signals used were A) tyrosine: 7.2 B) 4-HPP: 7.13, C) *cis*-acotinic acid 7.04, D) narciclasine 6.72, E) haemanthamine: 5.96 and F) sucrose: 5.44. Two-way ANOVA with Dunnett's test was used to compare samples from the same

harvesting time with the control samples (7 biological replicates, significance at (**a**) *P* < .05, (**b**) *P* < .01 and (**c**) *P* < .001, error bars represent standard error (SEM).

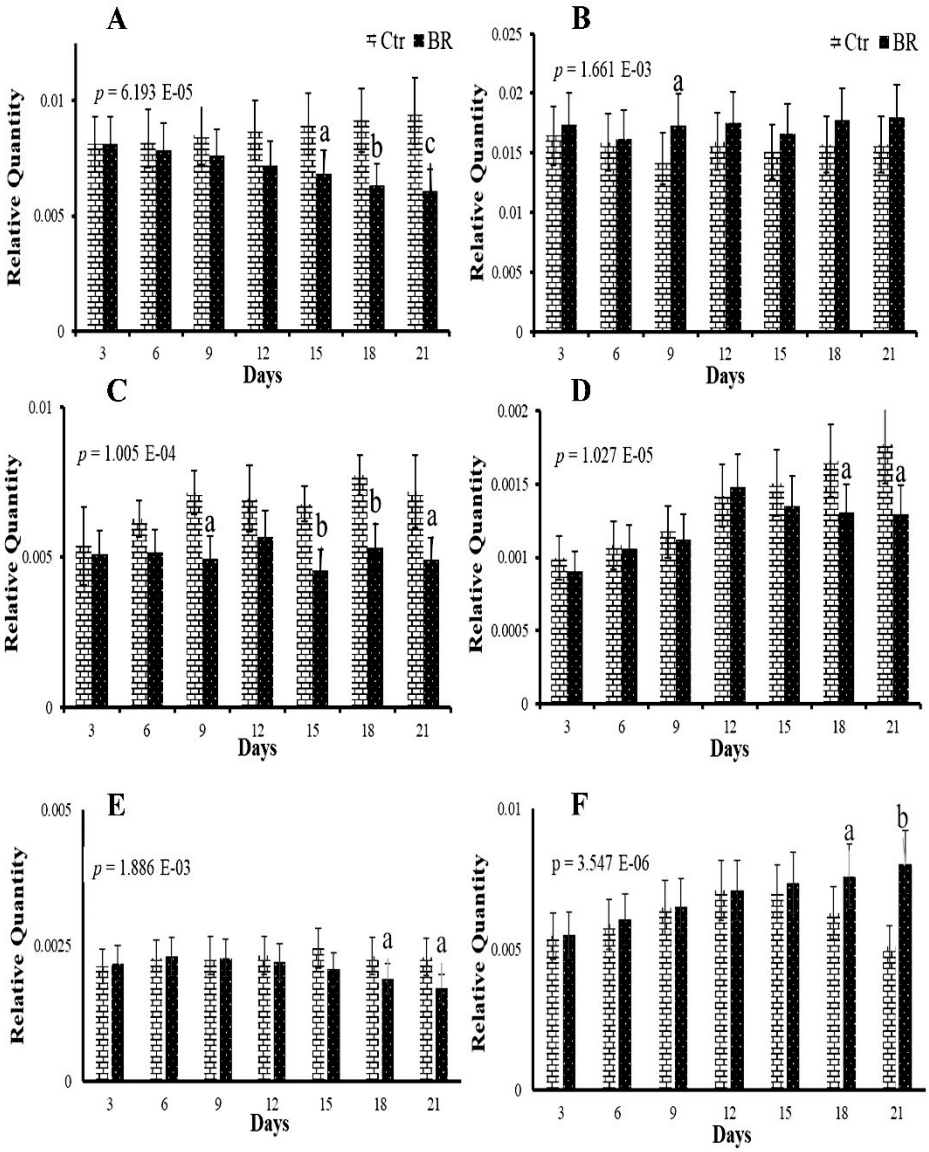

Figure 8.5. Relative molar quantification of compounds *Narcissus pseudonarcissus* controls (Ctr) and basal rot bulbs (BR) calculated from areas of ^{1}H-NMR signals (500 MHz). Chemical shifts of signals used were A) glucose: 5.20, B) asparagine: 2.95, C) choline: 3.21, D) ethanolamine: 3.12, E) alanine: 1.49 and F) fatty acids 1.31. Two-way

ANOVA with Dunnett's test was used to compare samples from the same harvesting time with the control samples (7 biological replicates, significance at (a) $P < .05$, (b) $P < .01$ and (c) $P < .001$, error bars represent standard error (SEM).

To identify the spectral areas responsible for the separation between control and infected samples as well as time after infection, samples were subjected to a SUS-plot. SUS-plot is a model which is used to solve the problem of multiclass complication by combining two different OPLS models and giving an overview of the effect of different classes to provide information about unique and shared traits. Based upon the two OPLS models (control-infected, time after infection) spectral areas were identified and assigned to corresponding metabolites (**Figure 8.3**D). It is evident from the results that amounts of phenolics including alkaloids were higher in the samples where infection rate was high while organic acids and amino acids were high in the samples with little or no infection.

With the help of multivariate data analysis (MVDA), the complexity of the data is reduced and maximum variance due to a factor can be seen. In the current study, MVDA shows that samples with little or no infection are clearly separated from the highly infected samples. To better understand the effect of infection of bulbs at different growth stages, some metabolites in infected samples were relatively quantified and compared with the control bulbs at the same stage. Well separated ^{1}H-NMR signal areas (calculated from bucket table) of these metabolites were used for their relative quantification (**Figure 8.4 and Figure 8.5**).

Selected signals of the compounds were subjected to two-way ANOVA with health and severity as the main factors. All the data could not be added here so an example of tyrosine analysis is mentioned here. From the statistical analysis it was clear that both main effects (health and infection severity) as well as their interaction had a significant effect on the relative quantity of tyrosine at the .05 significance level. The main effect of health yielded an F ratio of $F(1, 84) = 16.58375$, $p = 1.39$ E-08 which showed a significant effect. While the main effect of disease severity yielded an F ratio of $F(6, 84) = 15.5877$, $p = 4.44$ E-07 which showed a significant effect. At the same time, their interaction (type of cut × time after cut) yielded an F ratio of $F(6, 84) = 17.34188$, $p = 3.754$ E-08, indicated that difference in quantity of tyrosine were present when bulbs were infected and severity of the disease was varied. Post-hoc (Dunnett's test) revealed that highly severe disease (18

days) had the highly significant effect when compared with control sample at the same time point. While medium infected (12 days) condition had a significant effect when compared with the control sample at the same time point. It was evident from the statistical results that infection severity positively affected the tyrosine and its quantity was higher when compared with the control samples.

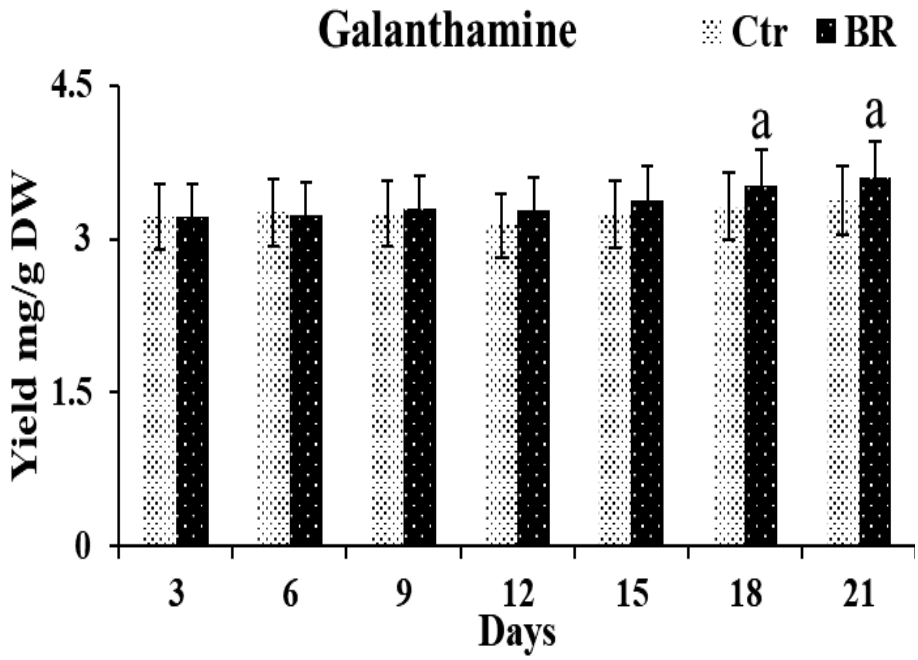

Figure 8.6. Galanthamine concentration mg/g dry weight in the infected samples of basal bulb rot compared with control at different stages of plant growth. Harvesting was done every 3rd day at the same time for control (CTR) and basal rot (BR) infected samples. Analysis of variance with Dunnett's test was used for comparison of galanthamine between healthy and infected samples at the same time of harvesting (7 biological replicates significance is at (a) $P < .05$, error bars represent standard error (SEM).

Similar analysis were performed for all the compounds which were mentioned in **Figure 8.4** and **Figure 8.5.** P-values of the interaction are mentioned in the figure with each compound. From the relative quantitation of metabolites, it was found that the alkaloids galanthamine, haemanthamine, narciclasine along with other metabolites such as asparagine, sucrose and fatty acids had significantly higher levels in basal rot infected

samples. At the same time, the levels of phenylalanine, glucose, choline, ethanolamine and alanine were significantly higher in the bulb samples where there was no infection or very little infection in the early stages of fungal attack. From these metabolites, galanthamine is the most important bioactive metabolite. In the current study, galanthamine contents in the bulbs were determined using the quantitative ^{1}H-NMR method described by Lubbe et al. [24]. After quantitation, the average amount of galanthamine (mg/g DW) was calculated from the biological replicates (**Figure 8.6**). From the statistical analysis it was clear that both main effects (health and infection severity) as well as their interaction had a significant effect on the total yield of galanthamine at the .05 significance level. The main effect of health yielded an F ratio of $F(1, 84) = 87235$, $p = .00243$ which showed a significant effect. While the main effect of disease severity yielded an F ratio of $F(6, 84) = 5.48235$, $p = .00379$ which showed a significant effect. At the same time, their interaction (type of cut × time after cut) yielded an F ratio of $F(6, 84) = 7.88526$, $p = .00203$, indicated that difference in quantity of galanthamine were present when bulbs were infected and severity of the disease was varied. Post-hoc (Dunnett's test) revealed that highly severe disease ($M = 3.5708 \pm SEM = 0.218$) conditions (days 18 and days 21) had the most significant effect when compared with control sample ($M = 3.3465 \pm SEM = 0.195$) at the same time point. From the statistical results it was clear that galanthamine contents were significantly higher in the bulbs which were severely infected with the basal rot.

Figure 8.7 shows ^{1}H-NMR spectra of bulb samples with different points of *Fusarium* infection compared with each other as well as with the control. On visual inspection of the NMR spectra, it is clear that some of the signals are different in both types of rots and the control samples. PCA analysis of control, neck rot and basal rot shows a clear separation of the samples in the PC1 vs PC2 plot (**Figure 8.8A).** It was clear from the figure that control, and infected samples were separated by PC1 and samples with a different point of infection were separated by PC2 which was an indication that certain metabolites were different between these three types of samples.

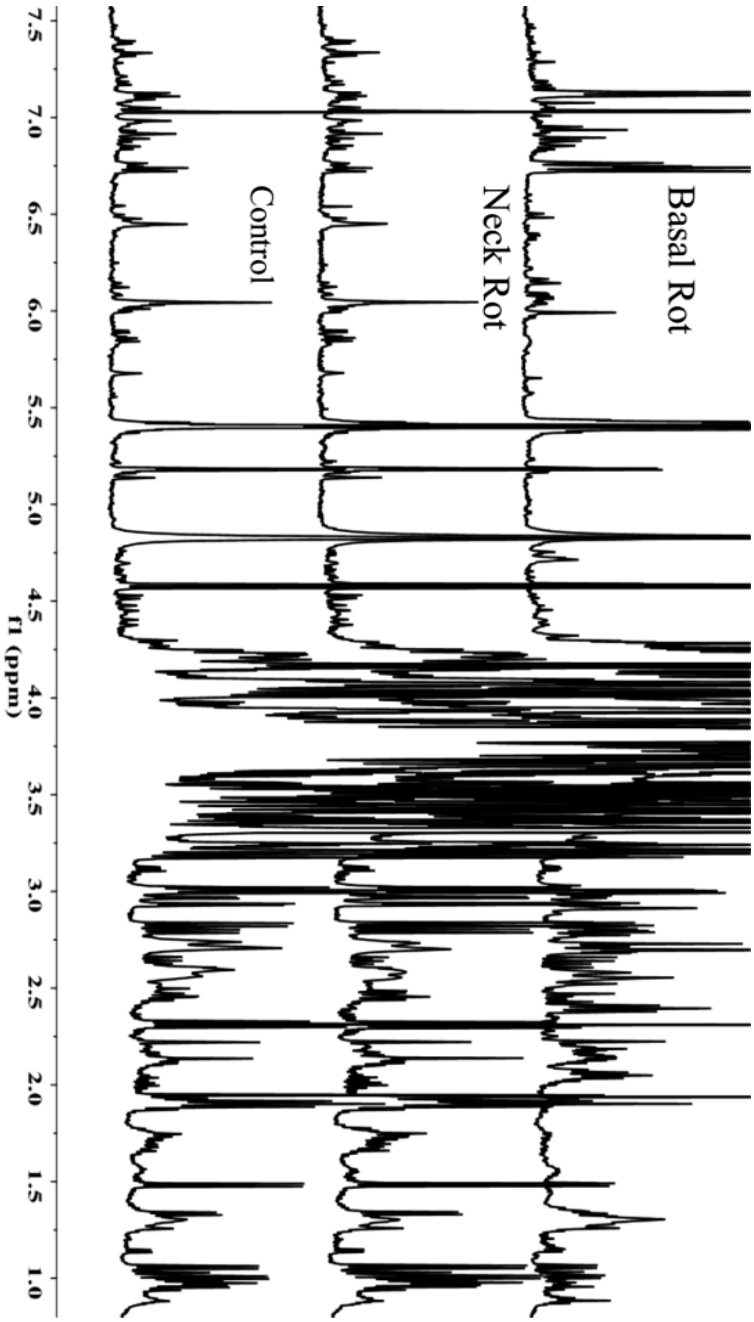

Figure 8.7. A) Comparison of ^{1}H-NMR spectra of extracts of *Narcissus pseudonarcissus* bulbs: control (Ctr), Neck rot (NR) and Basal rot (BR) infected bulbs at the time point when bulbs were fully infected.

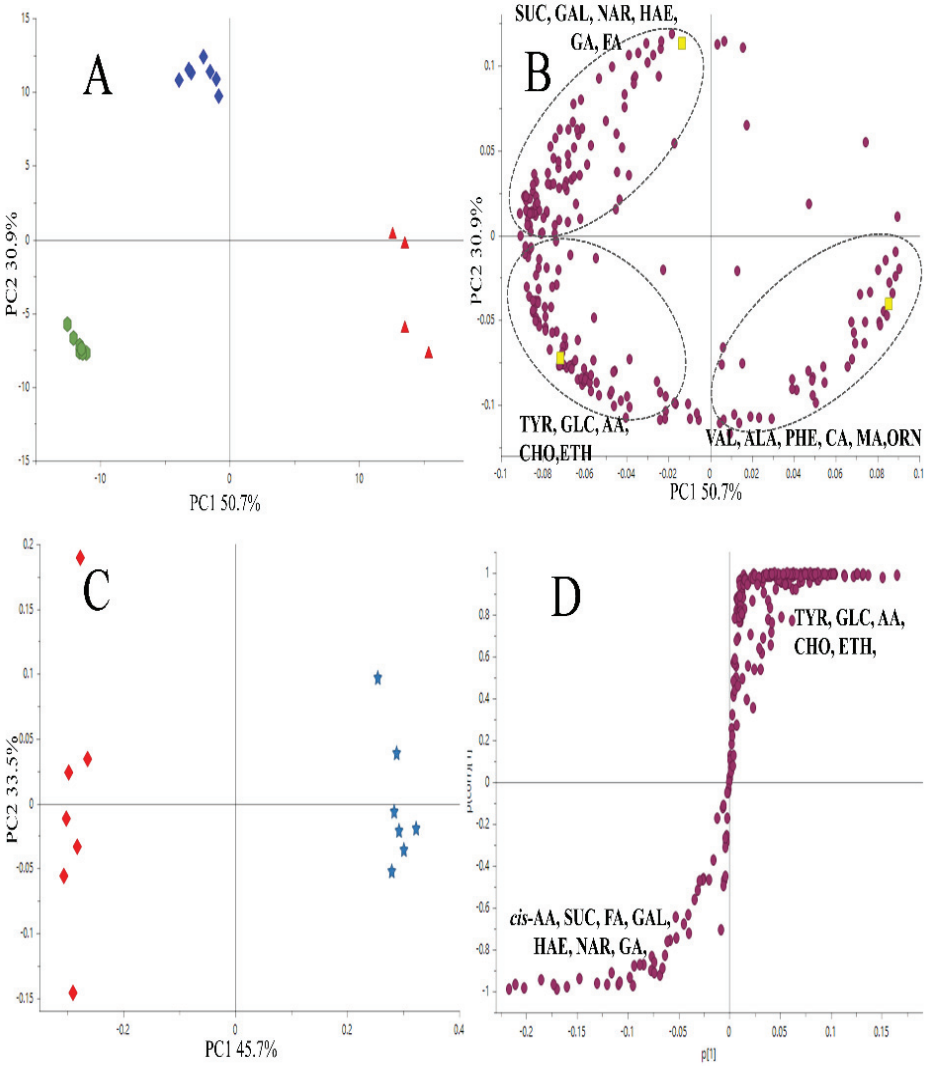

Figure 8.8. A) score plot of PCA (PC1 vs PC2) of ^{1}H-NMR spectra of extracts of *Narcissus pseudonarcissus* bulbs (●) control bulbs, (■) bulbs infected with basal rot and (▲) infected with neck rot.

B) Loadings plot of PCA valine (VAL), alanine (ALA), citric acid (CA), malic acid (MA), ornithine (ORN) sucrose (SUC), galanthamine (GAL), haemanthamine (HAE), narciclasine (NAR) and fatty acids (FA), tyrosine (Tyr), glucose (GLC), acetic acid (AA) and choline (CHO).

C) OPLS-DA score plot of infected bulbs where (●) represents basal rot samples and (○) represents neck rot samples.

(D) S-plot of OPLS-DA to determine the difference in metabolites connected with infection site.

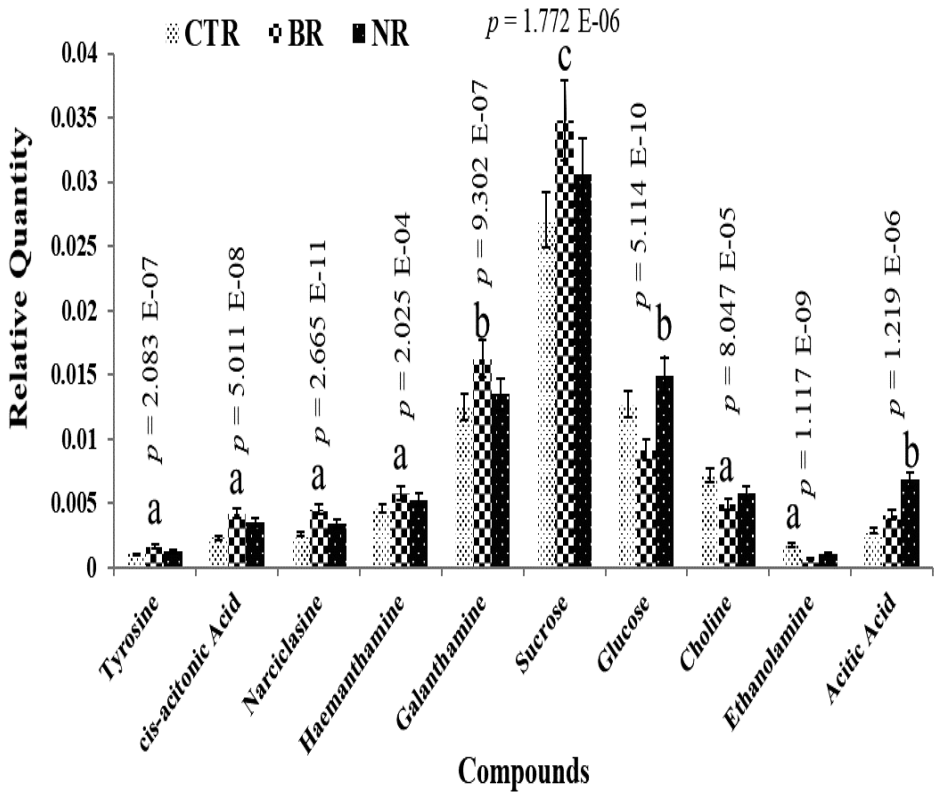

Figure 8.9. Relative molar quantification of metabolites calculated from signals of the ^{1}H-NMR spectra of extracts of *Narcissus pseudonarcissus* bulbs. Control (CTR), neck rot (NR) and basal rot (BR) infected bulb when fully infected. A one way ANOVA with Dunnett's test was used to compare samples of the controls and different rots (7 biological replicates, significance (a) $P < .05$, (b) $P < .01$ and (c) $P < .001$, error bars represent standard error (SEM).

To check the differences on the basis of point of infection, samples from neck rot and basal rot were subject to a S-plot (**Figure 8.8D)**. Loadings plot of the PCA and S-plot (**Figure 8.8B and Figure 8.8D**) clearly indicate that there were differences in multiple metabolites. Particularly the phenolics and sugars are the metabolites which cause the separation between both the rots and control samples. Relative quantification (**Figure 8.9)**

of some of the metabolites clearly indicate the differences between basal rot, neck rot and control samples. From the quantitative results, it is evident that the amount of tyrosine is higher in the basal rot infected samples than in neck rot and control.

Although sugars cause separation in both the rots, the amount of sucrose is higher in basal rot than in neck rot while the amount of glucose is higher in the neck rot samples than control and basal rot samples [25-29]. For alkaloids, there was a significant increase in galanthamine, haemanthamine, and narciclasine in both the rots as compared to the control samples. The more pronounced effect on alkaloids could be seen in the case of basal rot while in the case of neck rot the increase in alkaloid quantity was less than basal rot (**Figure 8.9)**. In the amino acids part of the spectra, there was a significant decrease in ethanolamine and choline in both basal and neck rot whereas a significant increase in the levels of glucose and acetic acid occurs in the case of neck rot.

Narcissus growers have been troubled since the 19th century by major losses in production during hot summers because of basal rot as this disease spreads rapidly during hot and humid summers. Some of the varieties are resistant to this fungus but commonly grown varieties such as Golden Harvest and Carlton are quite susceptible to this fungus thus suffering a lot of damage due to basal rot. During normal cultivation practices, the occurrence of this disease was related to increasing nitrogen fertilization in the field. In the early stages of the infection, there is a stilted growth of the stem and leaves of the plant but as the disease progresses it causes the bulb to rot. This rot starts from the basal plate inwards and upwards, which after some time causes the whole bulb to turn watery and soft. After this stage the bulbs become mummified. Though the neck rot is quite similar to the basal rot, it starts from the neck of the bulb instead of the basal plate and it moves downward in the bulb. **Figure 8.10** shows the spores of *Fusarium oxysporum* while bulbs at different stages of basal infection/rot are shown in **Figure 8.11**.

Although both rots are caused by the same fungus, the pathogenicity is quite different due to the location of the attack. In the case of basal rot, the damage is quick and very high while neck rots progress slowly and cause less damage than basal rot. Plants attacked by pathogens show several defense responses which include accumulation of some metabolites and a decrease of other metabolites.

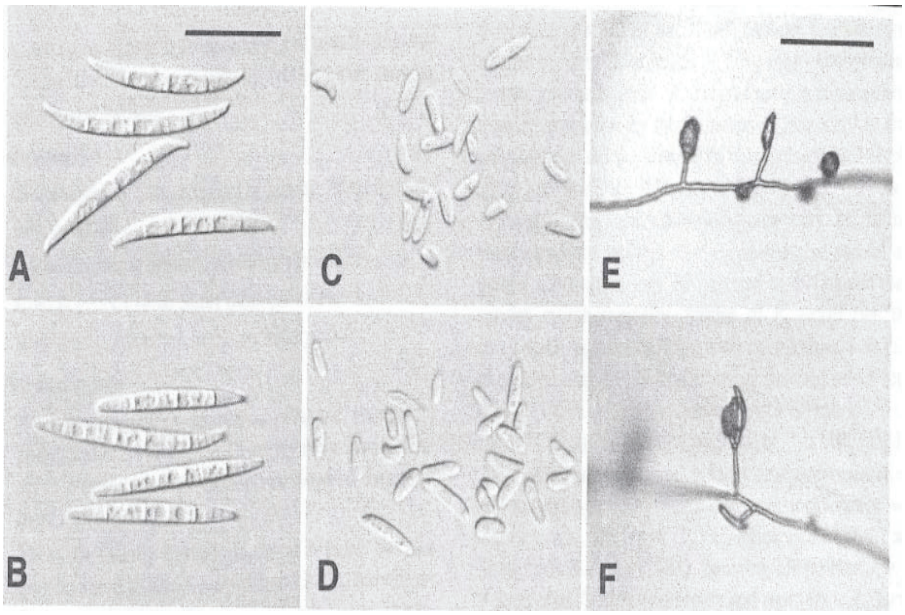

Figure 8.10. *Fusarium oxysporum* chlamydospores under different microscopic magnifications.

Figure 8.11. *Narcissus* bulbs at different stages of infection during basal rot.

The metabolites which often increase, or decrease are mainly secondary metabolites including alkaloids as many secondary metabolites are included in plant defense system. There are some reports that certain fungicides can cause the decrease in alkaloids in some plants [30] but in that case it was because an endophyte responsible for the production of the alkaloids was suppressed. Depending on their role in the plant alkaloid levels may decrease or increase in plants after infection. The effect of rot on the alkaloid levels from our results show a mixed response with reference to the mode of attack but there was a definite increase in alkaloid levels in both cases. The differences in alkaloid levels in comparison with the control are clearly shown by the NMR based metabolomics.

To confirm the MVDA results as well as alkaloid level increase and specifically of galanthamine, a targeted analysis of the ^{1}H-NMR spectra was performed. The results clearly showed that alkaloid levels in general were increased and the galanthamine level showed a significant increase when compared to the control bulbs.

In the case of haemanthamine and narciclasine, both alkaloids had significantly higher levels in infected bulbs than the controls. This increase in alkaloid levels can be attributed to the absence of roots in basal rot as in plant infected with basal rot there were no roots at all or only very short roots [31]. On the other hand, an increase of total alkaloids in general and galanthamine in specific might be attributed to a defense mechanism against the basal rot of the bulbs. In a study on *Narcissus*, the effect of fungicides on galanthamine content was checked and it was found that galanthamine was increased in the plants when a mixture of fungicides was applied and did not change or decrease when a single fungicide was applied in the field [21].

The fact that in both types of rots, an increase in alkaloid levels occurs if compared with the control could mean that these compounds are part of the plant defense against fungi or that fungus can affect the biosynthesis of these alkaloids. Moreover, a decrease in a number of amino acids, organic acids, and some sugars could mean that the fungus attack starts the use of these compounds in the plant to increase the production of defense compounds such as possibly galanthamine and other alkaloids. Some studies showed that high levels of sugars such as glucose, sucrose, and fructose are found in plants infected with *Fusarium* [23, 32]. In brassica leaves, the amount of sucrose was lower when the

plants were infected with *Fusarium*. Increased levels of sugars in infected plants may be due to an increased demand for energy and precursors for the biosynthesis of defense compounds. A decrease can be due to the disruption of the photosynthetic cycle or due to an increased sugar utilization for growth of the fungus and for the defense responses [25-29, 33]. From this study it is evident that as a farmer it is beneficial to remove the crop as soon as possible after seeing the symptoms for basal rot (stilted growth, no flowers) to obtain some income from the crop by using it as a source for galanthamine production otherwise the losses would be too severe, and bulbs would not be of any use.

This study illustrates how a ^{1}H-NMR-based metabolomics approach can be used to study an agricultural question. This method allowed the quantification of the compound of interest, galanthamine, while also providing quantitative information on metabolites of a variety of other chemical classes. Primary and secondary metabolites were identified and compared with the control plants to measure the difference in the production of these metabolites. Results clearly show that although the same fungus was responsible for both types of attacks in *Narcissus* plants, each type of attack generated a different response in terms of metabolites. Especially in the case of basal rot the fungal attack gave significantly higher levels of phenolics including galanthamine than in the control plants. Moreover, with the help of NMR, in a single measurement, a more wholesome view of the bulb metabolism could be obtained whereas multivariate data analysis provides a useful tool to simultaneously compare many variables between large numbers of samples. In terms of future prospects, it would be useful to test the response to fungal attack in the resistant varieties of *Narcissus* as well as the development of genetically modified fungus which can be used to trigger the plant defense mechanism and not completely destroy the crop in the process.

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