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Summary and Future Prospects

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Narcissus is an economically important crop with a large number of species, hybrids and/or varieties in cultivation. It is a very popular ornamental plant all over the world. Its importance as a floricultural crop came into view in the late 19th century and the importance as a crop has grown over time. According to the reports of consumer preferences in UK, *Narcissus* plants are ranked the second among cut flowers. It is quite an achievement considering the fact that daffodils are not available throughout most of the year and only obtained in a limited period of time, mostly during the spring season. The Netherlands is on the top of the list of *Narcissus* producers. Every year around 1700 hectares of *Narcissus* are planted in The Netherlands while in UK the area is around 1000 hectares. According to an economic report of 2009, annual Dutch sales of flowering bulbs including *Narcissus* reached up to 800 million Euros in total.

The ornamental values of *Narcissus* are well known but they are also a valuable resource of medicines, which is due to the presence of compounds, mostly species-specific secondary metabolites, such as alkaloids produced by the plants for their own defense against different external stress factors such as pests and diseases.

Among all these compounds of *Narcissus*, alkaloids are considered the most important due to their medicinal properties. So far from all the *Narcissus* alkaloids only galanthamine has been successfully commercialized. It is a tertiary isoquinoline alkaloid which reversibly inhibits the enzyme acetylcholine esterase (AChE). Due to this reason it is an important therapeutic agent for Alzheimer's disease (AD). Alzheimer's disease (AD) is a complex, neurodegenerative, multifactorial, progressive disease, mainly affecting the elderly people. It is estimated to account for 50–60% of dementia cases in persons over 65 years of age. The characteristic symptoms of this disease are loss of memory and impairment of multiple cognitive and emotional functions. The objective of this PhD research was to explain how galanthamine levels in the bulbs could be increased to make this floricultural crop to a medicinal crop.

In the 2nd Chapter of this thesis major biotic and abiotic factors, which can affect the production of galanthamine, are discussed. The factors also include different plant sources, which can be used for galanthamine production. Among all the plant sources

some *Narcissus* species give the highest yield of galanthamine per hectare. *Narcissus* is mainly grown at large scale for the flower market as well as for the sale of bulbs. Thus, there is a lot of information about the agronomic practices which are used for this purpose. But there is very little information on factors affecting the galanthamine production. For example, where and why galanthamine is produced by the plants is still unknown. It is important to learn more about the cultivation of the bulbs and learn what factors lead to the higher yields of galanthamine. In case of pests and diseases there is no information about the effect of these factors on galanthamine levels. So studies on the effect of diseases and herbivores are needed for better understanding of the plant resistance as well as the possible role of alkaloids in general and galanthamine accumulation specifically.

Whether the role of galanthamine is investigated in plants or the feasibility of commercial production of the compound is evaluated, the most important and basic step is to determine the amount of galanthamine in the plants and what plants can be used as a source material for production. For this purpose, methods were developed for the extraction and analysis of certain compounds. A number of methods have been reported for the analysis of galanthamine and these are reviewed in **Chapter 3**. These methods have been changed or improved over time following the developments of new types of equipment and in case of chromatography also new types of stationary phases. In earlier years, galanthamine analysis in plant material was based on thin layer chromatography (TLC) but shifted later to high performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC) with UV, diode array and mass spectrometry (MS) detection, gas chromatography coupled with flame ionization detector (FID) or MS, and nuclear magnetic resonance spectroscopy (NMR). Although the manual TLC methods are still in use for fast qualitative analysis, preference is now given to the quantitative instrumental methods using robotic procedures, for better reproducible and more robust results and higher sensitivity. The TLC is now mostly replaced by high performance liquid chromatography for the quantitative analysis of galanthamine in plants and biological samples. Still work on new method development is carried out to profit from the advances in the instrumentation and columns.

For quantitative analysis of certain target compounds HPLC with UV or PDA is mostly used. Concerning the detector in GC, FID is suitable for this purpose, with the advantage

of similar detection response for the analytes, where in the other detection methods large differences of several orders of magnitude may exist between different analytes.

The advent of the hyphenation of chromatography with MS, and NMR has led to changes in the way these tools are used. Particularly these methods now aim at an overall picture of all metabolites present in the biomaterial, i.e. metabolomics, instead of a targeted analysis. For highly complex mixtures the LC-MS becomes of interest as it adds an extra dimension plus higher level of certainty in identification of peaks. Metabolomics aims at identifying and quantifying the largest possible number of metabolites in an organism. Chromatographic methods in combination with MS as well as NMR are now the most widely used tools in metabolomics. In which the chromatography-based methods score best for number of compounds whereas NMR scores best for quantitation. Although NMR previously was used for the identification of a single pure compound, it is now used quite extensively for measuring extracts for metabolomics and metabonomics analysis because of simple sample preparation and fast analysis where in a single run with only one internal standard a large number of compounds can be quantified. NMR-based metabolomics has also been applied to the analysis of bulbs as well as for the quantitative analysis of galanthamine.

For the large-scale extraction of galanthamine conventional liquid-liquid extraction or solvent extraction methods are used mostly for plant materials. Further first purification steps are done by using centrifugal partition chromatography, or column chromatography. Super critical extraction can be used for more specific extractions. Microwave assisted extraction can improve speed and increase the yield from plant samples. A number of methods for the extraction of galanthamine on large scale have been published but the maximum amount which was obtained from the plants was typically about 0.12 % which is much lower than the amount present in the bulbs so there is still a lot of room for improvement in the process efficiency.

For our studies we needed the tools to quantify the various metabolites, and particularly the alkaloids. We thus investigated an optimum extraction method for galanthamine as well as the other alkaloids. Extraction/pre-analytical methods for the different techniques were evaluated. For this purpose (Chapter 4) a study was performed to check the effect

of amount of plant material (number of bulbs) from which a sample is taken for extraction. Moreover the weight of the sample required and the sonication time along with the amount of solvent which should be used for the best possible extraction of galanthamine from *Narcissus* bulbs were assessed, using $^1\text{H-NMR}$ analysis. The results show that the sampling pool has a significant effect on the reproducibility of galanthamine yield in the sample, but a high number of bulbs for only a small sample requires larger extraction equipment and solvent quantities, which from an environmental and economic point of view is not the preferred method. In terms of sample weight, there were little but no significant changes in galanthamine contents, although there were significant changes in the contents of other metabolites in general and phenolics specifically. The results from the sample/solvent ratio experiments showed that galanthamine yield was not significantly affected in absolute terms, but the relative quantities of other metabolites were. Moreover, saturation was observed for some compounds at increased sample to solvent ratios. For example, with the same amount of solvent, higher weight of the sample will result in the extraction of a relatively higher amount of carbohydrates and fatty acids than from lower weight samples. Concerning the amount of solvent required to extract galanthamine and other metabolites for analysis and quantification, the results showed that a slight change in the sample solvent ratio have a significant effect on metabolite production. It is evident from the results that 1.6 mL of solvent in comparison with 1.5 mL show a significant increase in metabolite levels. Here it should be noted that some metabolites might be inversely affected by the presence of higher amount of sugars or fatty acids in the extract thus quantity of these metabolites can decrease when there is an increase in the quantity of sugars and fatty acids. In terms of sonication time required for optimum alkaloid extraction, it was clear from the results that longer sonication time did have significant effect on the galanthamine contents although there was a slight increase in the temperature in comparison to the shorter sonication time. Thus after galanthamine quantitation and comparison it was concluded that 60-75 minute sonication time would be better for extraction of galanthamine and other metabolites for $^1\text{H-NMR}$ analysis.

The browning effect which is caused by the injury to the bulbs during the harvest can affect metabolite production. Browning is a process that occurs mostly in fruits and vegetables due to injury during the harvest or storage. The enzymes involved in this

process are polyphenol-oxidases. The effect of browning on galanthamine levels was used as a first application of the preanalytical and analytical protocol (**Chapter 5**). It was found that this process also affects *Narcissus* bulbs when they were exposed after cutting or injury. Results indicate that exposure area and time play an important role in the browning effect as bigger area and longer time will produce more enzyme. Although in terms of production of galanthamine and other alkaloids, longer time does not have much significant effect. It is clear from the results that maximum galanthamine production was obtained when bulbs were chopped and left for 15-30 minutes before extraction. Although all these pre-analytical processes have significant effect on the galanthamine production, it would be interesting to see the changes when they were all applied in different combinations to extract the maximum amount of galanthamine from plant material.

In the field conditions, flowers are cut from the plant as a normal practice either for the purpose to provide more nutrients to the plant and thus to obtain bigger bulbs for increased production or to sell the flowers in the market as a second source of income to lower the production costs. Thus, it was important to find out what effects are caused by the flower cutting in changing the metabolome of the plant in general, and specifically the galanthamine level. For this purpose (Chapter 6) flowers were cut from the plants grown in a greenhouse at different times of the plant growth cycle and in different ways in order to determine the best way and time to cut the flowers. Results indicate that flower cutting does have a significant effect on galanthamine levels and bulb growth. The best time for cutting the flowers to obtain better bulb yields as well as higher galanthamine contents was found to be as early as the bud emerging stage and as late as before the flower opening otherwise it would be too late to cause any significant effect in both bulb production and galanthamine levels. Because many bulbs are grown on saline soils close to the sea, the effect of salinity was tested on the plant growth and galanthamine levels. Salinity resulted in narrow leaves and early senescence of leaves thus causing a significant loss in the leaf mass, whereas it significantly increased the bulb production, thus affecting the total yield per hectare as well as causing a significant increase in galanthamine levels in comparison with the control samples.

A critical factor for galanthamine production is the age of the bulbs at the time of harvest. About 70% of all *Narcissus* bulbs are produced in The Netherlands and the UK, but the

cultural practices in both these countries vary a lot from each other. In The Netherlands, the *Narcissus* crop is lifted annually while in the UK it is a biannual process where half of the crop is lifted after a year while the other half is left in the field for two years for better production and to save production costs. Due to these reasons, the effect of the age of bulbs on the alkaloid contents was investigated (**Chapter 7**). Under normal conditions bulbs produce an outgrowth in the third year which can be separated from the bulb and used as a new bulb in the next year. Results clearly indicate that the annual harvesting is better compared with that of biannual. Although the two-year old bulbs have a higher amount of galanthamine as compared to the other bulbs but the yield per hectare is higher in the annual production system.

Moreover, the location for cultivation is important as different soils at different locations have different effects on the production of the bulbs and their metabolite contents in general and alkaloid contents in specific. As The Netherlands and UK are the two largest producers of *Narcissus*, bulbs produced in these countries were compared for alkaloid contents in general and galanthamine contents specifically with the help of NMR, GC-MS and GC-FID. The gas chromatography methods were used for the targeted alkaloids analysis. It was found out that bulbs produced in a certain place had significantly higher amounts of specific alkaloids while variations in the contents of galanthamine were not significant.

In terms of insects it is only the *Narcissus* fly that cause some damage in plants, but other diseases, particularly fungal diseases, cause a lot of damage to the *Narcissus* crop in the field. Basal rot is the one which causes most damage to *Narcissus*, it is caused by *Fusarium oxysporum*, a fungus which can survive under harsh conditions for many years in the soil. The effect of the fungal rot on the metabolites of *Narcissus* at different stages of infection as well as effect of point of infection has been studied (**Chapter 8**). For this purpose, *Narcissus* bulbs were planted in the soil infected with the fungus, and the bulbs were harvested at different time points after the leaf emergence when infection starts to take hold. From the results, it can be concluded that basal rot causes a significant increase in the accumulation of galanthamine and other alkaloids. At the same time it was also observed that with the progress of the infection, bulb yield was inversely affected which means that with a higher degree of infection, the losses in the bulb yield would be higher.

In comparing the point of the rot, it was clear that basal plate infection is quite damaging to the bulbs production when compared to the neck rot infection where losses were not that high. In terms of galanthamine levels on the basis of point of infection, a significant increase can be seen in the basal rot infected plants. Although neck rot also causes some increase in galanthamine levels, it was not significant in comparison with the control and basal rot infected plants. In general, it can be concluded that it would be beneficial for the farmers to harvest the crop as early as possible in case of basal rot infection and sell the bulbs for galanthamine production to cover their losses.

To study the effect of soil nutrients (**Chapter 9**) and for the improving bulb production along with galanthamine accumulation, water with different pH and humic acid were applied to the soil in which bulbs were grown. Although both of the treatments did not add extra nutrients, they affect the availability of the minerals and nutrients naturally present in the soil, which benefits the plants. From visual inspection of the plants it was clear that humic acid improved the plant growth as the leaves are greener and more broad and plant have more flowers per bulb, which consequently brings better quality of the plants. The galanthamine production was found to be significantly increased by the application of both humic acid and irrigation water with different pH.

From all the results it seems that galanthamine production can be improved by using specific procedures in the field during bulb production and some changes in the extraction process in the laboratory. From this thesis it can be generally concluded that a good place for bulb production as well as for high alkaloid contents is Lisse region, The Netherlands. The best age for highest galanthamine contents is around 2 years when the bulbs have maximum weight after the harvest from the field. For the highest yields per hectare the annual system is the best. During the cultivation of the bulbs, saline soils can be helpful in obtaining a higher bulb yield, thus making it possible to grow *Narcissus* bulbs in saline soils which are not fit for other crops. In terms of in field practices, double or triple application of humic acid in small amounts can be beneficial for bulb production in general and galanthamine production specifically. Moreover, the normal flower cutting field practice can be used before the flower opening stage in the field to augment the bulb production as well as significantly increase the galanthamine levels in the bulbs. Thus, considering all these factors, it can be said that galanthamine production can be

significantly increased by making some changes in the field practices and adding some additional treatments.

The most promising analytical tools used to monitor the galanthamine and general metabolite profiles in the *Narcissus* bulbs were ^1H -NMR spectroscopy alongside GC-MS and HPLC. These methods allow the quantitative analysis of galanthamine, while simultaneously providing qualitative and quantitative information on metabolites in other compound classes. Although a targeted HPLC-DAD approach was used for galanthamine identification and quantification, non-targeted approaches can also be used for specific metabolite analysis e.g. GC-MS and NMR. In the *Narcissus* studies, the limitations of the NMR method were mainly related to the relatively small number of metabolites detectable in the crude bulb extracts, and the extensive signal overlap which made signal identification challenging. Two-dimensional NMR experiments gave further resolution of signals and allowed identification of some further compounds. For the purpose of the present studies the target compound galanthamine could also be accurately quantified in the NMR metabolomics method. The combination of ^1H -NMR metabolomics data with that of other approaches may allow for better understanding of the observed effects. In the way it was used here ^1H -NMR analysis is an excellent analytical method for quantifying target compounds in plant extracts. As an approach to do metabolomics, as it was used here, it is a good first step for identifying pathways or groups of compounds for further investigations. Metabolomics is therefore a good hypothesis generating tool, but for answering deeper physiological questions additional information from complementary techniques is needed.

Future studies in the *Narcissus* field should focus on why plants produce galanthamine and thus learn how this can be manipulated to increase the amount of galanthamine produced by these plants. Existing crops being produced on large scale, as in the case of the ornamental bulb crops in The Netherlands, represent a good starting point for the search of novel compounds or products for industrial use. The fact that the plant material is already available on a large scale is a major advantage. It seems that for the yield per hectare particularly resistance against diseases especially against *Fusarium* would be an important aspect. There is also a need to breed varieties which can produce more galanthamine and have better bulb yields for large scale production of galanthamine.

However, the long time needed for breeding, 10-15 years for a new variety is a major hurdle in the specific breeding for alkaloid production. Moreover, concerning the biosynthetic pathway, identification of intermediates enzymes and genes is a must. The recent development in the integration of the omics technologies open the way to fully map the galanthamine biosynthetic pathway on the levels of genome, proteome and metabolome, including the molecular regulation of this pathway. That would open the way for metabolic engineering of the plant and even for a synthetic biology approach by introducing the whole pathway in other efficient production organisms, like bacteria or yeast. For breeding a multipurpose *Narcissus* should be the target that means a variety that can be used for the production of ornamental bulbs, flowers and medicines.