Ornamental bulb crops as sources of medicinal and industrial natural products

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General conclusions and perspectives

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Ornamental crops are produced in The Netherlands on a very large scale, and cut flowers and bulbs represent some of the country's major export products. Many of the ornamental bulb crops belong to plant families known to contain interesting bioactive chemical compounds. This means that in addition to the ornamental industry, these crops could potentially be cultivated for other industrial purposes. As a major part of this thesis the conversion of an ornamental crop, *Narcissus pseudonarcissus* cv. Carlton to a medicinal crop for the extraction of galanthamine was studied. Following a description of the cultivation of this ornamental crop in The Netherlands, as well as the quality expectations from the pharmaceutical industry, key points critical to the production of high quality raw material for galanthamine extraction were identified.

In Chapter 3 an overview is given of the general aspects that are important to consider in a Good Agricultural Practice (GAP) cultivation scheme. The most important part is the documentation of every step carried out in the production of the plant material, to ensure complete traceability. An example of a document that could be used in such a cultivation scheme for *Narcissus* bulbs is provided in Appendix A. Some key points in the cultivation process were identified that could affect the quality of the bulbs. Experiments were conducted to investigate these key points so that recommendations can be made for a cultivation scheme that would be suitable for GAP.

The first critical step to be considered was the pesticide treatment of the bulbs before planting. The results of a field study showed that the galanthamine levels of bulbs treated with certain fungicides before planting were significantly different from a control treatment. Compared to the control treatment, the pre-planting fungicide treatment most closely representing that as typically done in ornamental cultivation had the highest galanthamine concentration (although not significantly higher than the control). The metabolomics analysis showed that fungicides with different modes of action can cause different alterations in the plant metabolism, still detectable in the bulbs at the end of the growing season. Pesticide application in the field was the next point to consider in the *Narcissus* production chain. Here effects were again seen in the galanthamine levels and metabolite profiles as compared to the control treatment.

Generally lower galanthamine levels were seen, and altered sugar metabolism was also observed. However, when in-field fungicide treatment was combined with an application of mixed fungicides before planting, no lowering of galanthamine was seen. These results showed that while individual fungicide applications could lower the galanthamine levels, the fungicide mixtures typically applied in the production of *N. pseudonarcissus* cv. Carlton does not have a negative effect on the galanthamine in the bulbs. In a screening study of pesticides and herbicides applied before planting and in the field, some residues were detected in the plant material. If fungicides and other protective agents (herbicides, insecticides) are applied during cultivation, this should be documented so that targeted analysis of residues can be conducted. Another step investigated for its effect on galanthamine in the bulbs was the application of fertilizers in the field. The standard nitrogen and potassium fertilizer application as done in ornamental cultivation was found to be optimal for production of galanthamine in the bulbs. Applying more nitrogen or potassium fertilizer did not result in further increases of the alkaloid. Using a metabolomics approach it was seen that various primary metabolite pathways were altered upon increased fertilizer treatment.

Fungal infection, in particular with *Fusarium oxysporum* is a major problem in the cultivation of *Narcissus* in the Netherlands. While it may result in lower yield of bulbs, the possibility exists that the fungi may produce mycotoxins in the bulbs. Bulbs infected with this fungal pathogen were screened for mycotoxin content, and the presence of one, beauvericin was confirmed. This compound is toxic to human cells and thus only healthy bulbs should be used for galanthamine extraction. Since it is not always obvious that bulbs are infected with *F. oxysporum*, targeted screening for beauvericin may be a necessary quality control step as part of a GAP scheme.

Time of harvest may influence the galanthamine levels in the bulbs. Previous pot studies and field studies done in the UK showed that galanthamine in the bulbs were at a maximum around the time of flowering. In a field study monitoring galanthamine over the course of a growing season galanthamine level was found to reach a maximum before flowering. For sustainable production of the bulbs for galanthamine extraction, harvesting at the end of the growing season as for ornamentals is a better option, as this will ensure a good yield and bulb planting stock for the next season. In addition to galanthamine, the changes in the next two most abundant alkaloids, haemanthamine and narciclasine in the leaves, bulbs and roots were also investigated. While not as abundant as galanthamine, the bulbs represent a potential source of these two interesting bioactive compounds. Their extraction from the bulbs in addition to galanthamine could add further value to the bulb as raw material for industrial use.

In the final part of this thesis the discovery of a bioactive small molecule, 6-tuliposide B, in the gum produced by tulip bulbs is described. This was the result of investigations into various ornamental bulb crop materials for compounds for potential industrial use. The process of gummosis is a physiological response to fungal infection, which can also spread to otherwise healthy bulbs in storage due to the release of ethylene. This response is undesirable as it results in lower bulbs yields. The idea was to investigate the small molecule profile of this unwanted "waste" material for interesting components. The presence of tuliposide in the gum at high purity as well as the fact that the gum can be induced in healthy bulbs made this an interesting potential production method of this compound. Studies were carried out to investigate the occurrence of 6-tuliposide B and related compounds in gums of various tulip cultivars. The process of gum induction was also studied in experiments testing various factors that influence gum production and tuliposide content. It was shown that with some careful optimization it would be possible to produce tuliposide-containing gum from the bulbs of certain tulip cultivars for industrial use. From the amounts of gum that it is currently possible to obtain it seems that this would be most suitable for extraction of the small molecule for use as a fine chemical, as opposed to something needed in larger amounts (e.g. biopolymer building block).

The main tool used to monitor the galanthamine and general metabolite profiles in the *Narcissus* bulbs as well as the tuliposides in the tulip gum was ^{1}H NMR. This method was useful as it allowed the quantitative analysis of galanthamine, while simultaneously providing qualitative and quantitative information on metabolites in other compound classes. In the tulip gum it was very useful, as quantification was possible without a reference standard of the compound of interest. It was also possible to obtain structural information about related compounds in the gum. In the *Narcissus* studies, the limitations of the 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 were able to aid further resolution of signals and identification of compounds to some extent. For the purposes of the studies conducted, where the target compound galanthamine could be accurately quantified, the method was suitable. For deeper insights into the physiological processes and mechanisms behind the observed results further targeted metabolite analyses would be needed. The combination of ^{1}H NMR metabolomics data with that of other approaches would allow for better understanding of the observed effects. For example, gene expression studies or targeted biosynthetic pathway analysis could help explain why certain changes were seen in galanthamine and other metabolites in response to certain fungicide applications. In the way it was used here ^{1}H NMR analysis is a good analytical method for quantifying target compounds in

optimized plant extracts. As an approach to do metabolomics, as it was used here, it is a good first step for identifying a pathway or group of compounds to investigate for a given experiment. It is therefore a good hypothesis generating tool, but for answering deeper physiological questions additional information from complementary techniques is needed.

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. Also cultivated plants already have well-established cultivation practices, which saves time in comparison to wild plants which have to be brought into cultivation for the first time. The Dutch bulb crops have a further advantage, namely the existence of thousands of cultivars. This large genetic diversity is the result of centuries of hybridizations, and could potentially mean a large chemical diversity to be explored.