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Chapter 3. *Narcissus*: from ornamental to medicinal crop.

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Abstract

Daffodils (*Narcissus* spp.) are well known ornamental flower crops. They have been cultivated for their yellow trumpet-like flowers for centuries, particularly in the Netherlands where cultivation practices are well established. As other members of the Amaryllidaceae plant family, *Narcissus* plants produce various bioactive alkaloids. One of these alkaloids, galanthamine, has been developed and licensed as a drug against symptoms of Alzheimer's disease. An increasing demand for the drug and economic and sustainability issues related to its supply creates the need for alternative sources of galanthamine. This created an opportunity for *Narcissus* growers in the Netherlands to convert their ornamental crop to a medicinal crop. Studies were initiated to assess the feasibility of producing *Narcissus* bulbs as raw material for galanthamine extraction. In order to produce raw material for pharmaceutical extraction, it is important to know which quality standards exist and what is expected from the buyer of such material. This chapter provides an introduction to Good Agricultural Practice for the cultivation of medicinal plant material. The alkaloid galanthamine is introduced, and an overview is given of how *Narcissus* cultivation is typically done for ornamental purposes. This provides a background for the following chapters in which key points critical to the production of high quality raw material for galanthamine extraction will be investigated.

Good Agricultural Practices for cultivation of medicinal plant material

In recent times awareness of and concerns over the environment and the sustainability of agricultural methods have increased. Globalization and international trade has also brought producers and buyers that have vastly different agricultural practices and standards into contact. Good Manufacturing Practices (GMP) for processed goods (e.g. processed foods, pharmaceuticals) have been developed and have become routine parts of business protocols and national and international government policy regulations. This has led to various agencies and organizations (e.g. FAO, WHO) developing recommendations for producing raw materials for use in GMP processes that will be of an appropriate standard and quality. The application of Good Agricultural Practices (GAP) was thus developed as a way to ensure the safety and quality of the whole chain, from production of raw material to final product.

Good Agricultural Practices are a collection of principles to apply for on-farm production and post-harvesting processes, resulting in safe and healthy food and non-food agricultural products, while taking into account economic, social and environmental sustainability. The GAP process embraces actions, technologies and systems that are accepted as the most effective for optimal management of soil and water, and for crop production, from the point of view of microbiological and chemical safety, together with environmental, economic and social sustainability. Details of a GAP protocol for a commodity in a given production environment cannot be generalized, but must be based on general underlying principles and norms while adapted for the local situation. Common principles of GAP include describing farming that uses available technology optimally to promote agricultural productivity of safe and healthy crops, to achieve economic viability and agricultural, environmental sustainability and social responsibility. As described in chapter 1, GAP recommendations were initially set up for food crops, but additional recommendations have been formulated for medicinal plant crops.

The scope of a given GAP scheme should reflect its intended objectives, which can include some or all of the following: ensuring safety and quality of produce and allowing traceability, minimizing environmental impact and creating product differentiation (e.g. in order to capture new market niches). Cultivation systems are not yet in place for many medicinal plants, and if new ones are to be developed it can be done according to specific aims following these guidelines. The following section will try to give a brief overview of the type of information that may be required so that such a production system can be set up in practice. A general overview is given for producing raw plant material for medicinal or pharmaceutical use. Thereafter a specific example, namely the cultivation of *Narcissus* plants (daffodils) as a source of the alkaloid galanthamine will be discussed in detail.

Quality assurance

An important aspect at the beginning of the GAP process should be defining what is meant by quality. An agreement on what is expected by buyers of medicinal plants/herbal substances from the producers should be laid down in writing, and should comply with recognized regional and/or national specifications. This would be regarding content of active constituents, limit values of chemical residues, heavy metals and microbial contamination. In cases where a whole plant will be used it can be challenging to set standards for active compounds, as often it is not precisely known which of the many metabolites are responsible for the medicinal effect. If one compound is to be extracted from the plant material this may be easier to determine and monitor.

Documentation

An important aspect of GAP is traceability. All processes and procedures carried out during cultivation that could affect the quality of the product should be documented. This may be used in audits to ensure the buyer that the terms agreed on before were adhered to. GAP protocols require documentation to be kept for a certain minimum number of years.

Selection of medicinal plants and botanical identity

Good Agricultural Practice recommendations state that the plant species or variety to be grown should be chosen according to the country's pharmacopoeia, or what is specified in the end-users country's equivalent authoritative documentation. This is assuming that it is a well-known plant with monographs prepared on its medicinal use. If not, research should be carried out to determine the species, cultivar or variety with the most suitable make-up, in terms of chemical constituents and disease resistance. It is very important that the botanical identity of the cultivated plant should be verified. The scientific and common names should be recorded, as well as other relevant information such as chemotype, phenotype and cultivar. All origins of seeds, plants or propagation material should be well recorded. If the plant of interest is not well known, or if there is doubt about its identity, voucher specimens should be submitted to a herbarium for identification. Comparing the genetic pattern of the specimens to an authentic one can confirm identity.

Seeds and propagation materials

It is important that the plant material produced can be traced to its source. The origin of seeds and propagation material should be specified. Commercial suppliers of materials should as far as possible provide information of the identity, quality and performance of the product, as well as breeding history where relevant. Seeds and materials should be as free as possible from contamination and diseases for healthy plant growth. Any treatment of seeds (e.g. with agrochemicals) should be documented. The quality of planting or propagation materials should comply with regional or national regulations, and all necessary labels and documentation must be present.

Cultivation

If no cultivation method has been established, traditional methods of growing the plant can be followed. Otherwise research should be conducted to develop appropriate methods. Information about the effects of water, nutrients, light and temperature on the secondary metabolite composition of the plants will be needed. Also studies to determine the best planting arrangement in the field will be needed. The presence of weeds in the field should be documented. Weeds can potentially affect the growth of the crop, and can potentially be a source of toxic compounds (e.g. pyrrolizidine alkaloids) when accidentally harvested together with the bulbs. The effect of stress factors (e.g. salt, water, and drought) that may occur should also be studied to see how it would affect the secondary metabolism of the plant. During cultivation any extraordinary conditions or circumstances during growth of the plants should be documented, as it may affect metabolite composition of the plant. Further details of cultivation will depend on whether organic or conventional methods are followed, and on the quality of the product required. Principles of good husbandry should always be applied throughout cultivation, for example the appropriate rotation of crops, and adoption of soil tillage to plant growth and requirements. At all times it should be kept in mind that the environmental impact should be minimized.

Soil

The soil on which plants are grown can have a large effect on the growth and metabolism of the plants. The site chosen to grow medicinal plant crops should contain the appropriate amounts of nutrients, organic matter and other elements for the specific plant. If optimum conditions are not known, trials can be performed to see how soil conditions influence growth and quality. All soil parameters should be documented (e.g. pH, water content, nutrient content, intensity of sunlight, temperature). Additional information about the history of the site should be recorded, such as previous crops grown there, chemicals used before and any contamination with diseases or pests. Fertilizers should be used sparingly, and careful research should be carried out to determine the correct types and quantities needed. Keeping in mind environmental impacts, fertilizers should be applied in such a way as to reduce leaching from the site of use. If manure is used to fertilize the soil, it should be properly composted, and no human feces may be used. Further soil parameters to take into account are microbial populations in the soil. A healthy mycorrhizal population may have a great impact on health and quality of the plants grown. Monitoring and maintaining microbial populations may therefore be a part of the protocols of the production chain.

Irrigation

Irrigation should be controlled and carried out according to needs of the particular medicinal plant at various growth stages. It is important that the water used should meet national quality standards, for example be free of heavy metals and microbial contamination.

Crop protection

As a rule agrochemicals to promote growth or protect plants should be kept to a minimum. Integrated pest management (the integration of a combination of all available pest control techniques to discourage pest populations and minimize pesticide use and risk to the environment) should be followed where appropriate. Herbicides and pesticides used should be in accordance with recommendations of manufacturers and authorities, not only in the grower's country but also in that of the end-user. Levels of agrochemical residues in the plant material should be checked and reported. If methods to do so are not available they should be developed. Maximum pesticide and herbicide residue limits as described by regional or national regulatory authorities in grower and buyer's countries should be adhered to. Only qualified staff should be allowed to apply agrochemicals, and all applications must be documented. Applications should be in accordance to manufacturers' instructions, and should be carried out with the agreement of the buyer. There have been reports of pesticides and herbicides affecting the metabolism of plants (Deng 2005, Garcia et al 2001, 2003), and this can have consequences for the quality of a medicinal product. If such chemicals are used, studies can be conducted to check the effect of these on the target compound(s) on the metabolome of the plant.

It should be known which pests and diseases are most likely to affect plants in a cultivation system. This will allow the more directed and precise use of agrochemicals. A particular pest may have a significant effect on plant quality. For example attack by certain insects or pests may result in elicitation of defense compounds, which may be desired in higher amounts in the plant material. Attack or infection by other pathogens or pests may cause the presence of unwanted metabolites in the plant material (e.g. mycotoxins, plant defense compounds).

The use of herbicides should be controlled and appropriate ones used according to the plants that are most likely to grow as weeds with crops. Application should be done carefully to minimize impact of herbicides on non-target plants. Control of weeds are especially important where medicinal plants are grown to be used in crude form, as adulteration and the accidental inclusion of undesired plants can more easily occur if weeds are present at harvest.

Harvest

Time of harvest is very important for medicinal plants as it is well known that concentrations of active compounds vary during growth stages. The optimal time (time of day/year) will depend on the particular species. Information about this can be found in pharmacopoeia, published standards and monographs, or if not available research should be conducted to determine it. Harvest time is not only important for desired compounds in the plant, but also for the presence of potentially toxic and otherwise unwanted non-target compounds. As mentioned before care should be taken when harvesting not to include any foreign substances or unwanted plants or weeds.
As in all other steps in the GAP process, all important parameters related to the harvesting should be documented. These include date and time, and also the weather conditions of harvesting. Harvesting should be avoided on days where conditions are favorable for molds or microbial fermentation to occur, for example on very humid or wet days. Care should be taken upon harvesting to avoid mechanical damage of the plant material. Damage caused by compacting or overfilling of boxes for example can have an undesired effect on the quality of plant material.

Primary processing

After harvesting plant material should be inspected for possible substandard material and for the presence of foreign substances. Depending on the plant material, some primary processing might need to be carried out such as washing, drying, cutting, fumigation, freezing etc. Specific processing may need to be done to improve the purity of the plant part being used, reduce drying time, to prevent mold damage, detoxify toxic ingredients and enhance therapeutic efficacy. Examples of such processes include: peeling skins of roots and rhizomes, steaming, soaking, pickling, distillation, fumigation, roasting, treatment with lime, chopping, and natural fermentation. Any processing performed should be documented and possible effects this can have on the quality should be investigated. Since processes like cutting can alter the chemistry of fresh plant material, reproducible methods should be developed to ensure consistent quality. Reproducible drying methods should also be developed. For example, if plant material is grown for essential oil content, any drying processes should be done carefully to preserve the volatile components. Plant material should be packaged and stored to avoid interference from pests. Conditions of storage should be carefully chosen to preserve desired quality of the plant material, and should be well documented. Studies may be conducted to determine optimum conditions.

Cultivation of *Narcissus* as raw material for production of Galanthamine

Narcissus plants are cultivated in the Netherlands as ornamental plants. The discovery that they produce alkaloids with useful medicinal properties aroused interest in the cultivation of *Narcissus* as a medicinal crop. Cultivation of ornamental crops is different from that of crops to be consumed as food or medicine. For ornamentals yield and visual aspects of the plants are most important, and often a high input of agrochemicals is needed to satisfy these needs. Yield is also important for medicinal crops, but more important are quality aspects such as efficacy and safety. Efficacy is related to the levels of active compounds in the plant material. Safety involves the absence of toxic and potentially harmful contaminants or metabolites produced by the plant. The principles described above aim to satisfy these needs in a medicinal crop. In the following sections some aspects that may be important in the cultivation of *Narcissus* as a medicinal crop will be discussed.

Introduction to Galanthamine

Galanthamine (4a,5,9,10,1,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2,-ef] [2]-benzazepin-6-ol) is a natural product, belonging to the class of compounds called alkaloids. It is a benzazepine alkaloid belonging to the Amaryllidaceae group, which is found in the plant family of the same name. The structure of Galanthamine is shown in Figure 1. Galanthamine has become an important compound due to its chemical and biological properties. It is a small molecule that upon ingestion is able to cross the blood-brain-barrier and act in the central nervous system (Mucke, 1997). It inhibits the enzyme acetylcholinesterase in a long-lasting, selective, reversible and competitive manner (Moghul and Wilkinson, 2001). Acetylcholinesterase (E.C. 3.1.1.7; AChE) is one of two cholinesterase enzymes that occur in the human central nervous system. It hydrolyses acetylcholine at cholinergic synapses to terminate nerve impulse transmission. Inhibition of AChE activity increases the availability of acetylcholine to sustain nerve cell communications, and drug discovery efforts have searched for chemical inhibitors as possible leads for nervous system disorders (Lenta et al., 2008). Inhibition of AChE has been a strategy for treatment of senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Mukherjee et al., 2007), but probably the most attention has been given to finding AChE inhibitors to treat Alzheimer’s disease (AD).

AD is a chronic and progressive neurodegenerative disease, and is the most common cause of cognitive decline in elderly humans (Akhondzadeh and Abbasi, 2006). Apart from cognitive decline and memory loss, other symptoms of the disease include depression, behavioral problems and language deficits (Howes et al., 2003). Pathological features that characterize the disease are extracellular amyloid-ß-peptide deposition in senile plaques, neurofibrillary tangles, and degeneration of the basal forebrain cholinergic neurons. The loss of cholinergic neurons causes a significant reduction in acetylcholine, and this cholinergic deficit has been found to play an important role in the cognitive impairment associated with AD (Bartus et al., 1982). Increasing the levels of acetylcholine in the brain by inhibiting AChE has become the most extensively used strategy for the symptomatic treatment of AD.

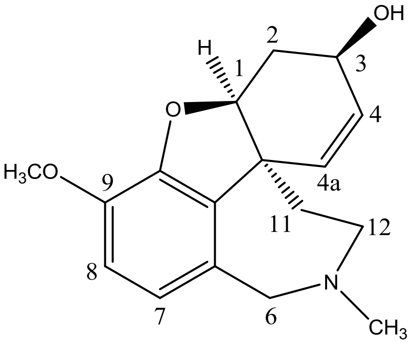

Figure 1. Chemical structure of galanthamine.

There is currently no preventative or curative treatment available for AD. Providing relief of the symptoms of the disease is the only available therapeutic option. Galanthamine is used therapeutically as the hydrobromine salt, and offers superior pharmacological profiles and fewer side effects than other AChE inhibitors such as tacrine and physostigmine (Giacobini, 1998). It causes short-term improvements in cognition, function and in general every day activities for patients with mild to moderate symptoms, but it is not known whether it is effective in the longer term, after about 6 months (Patterson et al., 2004). Apart from the AChE inhibitory activity, galanthamine also possesses other biological activities. It is known to stimulate pre-and postsynaptic nicotinic receptors which can increase the release of neurotransmitters, and possibly protect against B-amyloid toxicity (Scott and Goa, 2000; Sramek et al., 2000; Arias et al., 2004). Other pharmacological actions not directly related to AD were summarized by Bastida (2006), and include affecting membrane ionic processes, causing bradycardia or atrioventricular conduction disturbances, ability to reverse aneshesia and inhibition of traumatic shock. Galanthamine has been patented as a smoking-cessation agent (Moormann, 1997), it can relieve jet lag, fatigue syndrome, male impotence, alcohol dependance, and can reduce intraocular pressure. Furthermore it acts as a hypotensive, has weak antimalarial activity, acts as a mild analeptic and has analgesic properties.

Galanthamine was first isolated from the snowdrop plant, *Galanthus woronowii* (Heinrich and Teoh, 2004) . The use of this plant in traditional medicine systems of the eastern European regions where the plant is native has been mentioned, but clear records of this are not easily found. It is said that a Bulgarian pharmacologist noticed people rubbing the plant on their foreheads to relieve nerve pain, and thereby decided to investigate the plant further for medicinal use (Bastida et al., 2006). The first paper on pharmacology of Galanthamine was published in 1951(Mashkovsky and Kruglikova-Lvova, 1951), which showed the AChE inhibitory activity as well as the alkaloid's ability to reverse curare-induced paralysis. In the 1950s various pre-clinical investigations into pharmacological effects of galanthamine continued, and eventually the compound was registered under the trade name Nivalin® and became commercially available in Bulgaria. At the time the main use for Galanthamine as a medicine was to treat post-poliomyelitis paralysis, and other neuromuscular diseases (Bores et al., 1996; Radicheva et al., 1996). In the 1980s researchers into AD developed the "cholinergic hypothesis", which proposed a central role of cholinergic function in the pathology of the disease (Heinrich and Teoh, 2004). In 1986 galanthamine was applied to AD for the first time (Heinrich and Lee Teoh, 2004). Promising results lead to clinical trials of galanthamine for the treatment of AD, and in 1996 an Austrian company, Synochemia Pharmazeutika launched it as Nivalin®. Later two companies, Shire Pharmaceuticals in Britain and Janssen Research Foundation in Belgium, took the development of galanthamine further and launched Galanthamine as Reminyl® in a larger number of countries (Sramek et al., 2000; Heinrich and Teoh, 2004). Since then clinical studies have continued to be done and much research attention is still focused on this alkaloid for the use in AD and other indications.

Sources of Galanthamine:
As mentioned before, galanthamine was first isolated from the plant *Galanthus woronowii*. Other members of the Amaryllidaceae family have also been found to contain this alkaloid, most notably *Leucojum aestivum* and *Narcissus* species (Heinrich and Teoh, 2004). For about 40 years, *Leucojum aestivum* was the main source of galanthamine in Eastern Europe. With the more widespread licensing of Galanthamine in the western world in recent years, alternative sources to the quite limited supply from *Leucojum* have been investigated. In the 1950s the genus *Narcissus* was investigated as a source of galanthamine, and it was found in various species (Cherkasov, 1977). Levels of the alkaloid vary between cultivars and species, from only trace amount to as much as 2.5 % of dry weight (Cherkasov and Tolkachev, 2002). A full chemical synthesis of

galanthamine was published in 1962 (Barton and Kirby, 1962), but this biomimetic method was for proof of structure and had a yield too low to be useful for commercial purposes. Since then many synthetic methods have been published to improve on the original ones (Lewis, 1998, 1999, 2000, 2001; Jin, 2003, 2005). Many methods now exist for the synthesis of galanthamine, either total synthesis or from precursors. The first patent on the synthesis of galanthamine was obtained by Sanochemia Pharmazeutika in 1996, with global protection of the process. Cooperation agreements with Janssen (Johnson & Johnson) and Shire Pharmaceuticals followed a year later (Heinrich and Teoh, 2004).

Narcissus as raw material for Galanthamine production

Recently synthetic methods have been used to produce galanthamine for the pharmaceutical industry (Tiffen, 1997). The high cost of production was acceptable because of the protection offered by licensing and patents, giving companies exclusive rights to produce the drug and so having no competition where the price is concerned. Patent protection expired recently, allowing generic products to enter the market which can bring drug prices down. Also, in 2004 about 15 million people were estimated to be suffering from AD worldwide, and this number is predicted to be three times higher in fifty years' time (Forman et al., 2004). The increasing demand and the impending price competition make other sources of galanthamine attractive. Focus has again shifted back to *Narcissus* plants, and specifically the bulbs, as a source of galanthamine. In the UK a large study into the production of Galanthamine from *Narcissus* was launched by the Department of Environment Food and Rural Affairs. The cultivation of *Narcissus* plants as raw material for the production of Galanthamine in the Netherlands is now being investigated.

Cultivation of *Narcissus* in the Netherlands

Introduction to *Narcissus*

Narcissus L. is one of 59 genera in the monocotyledonous family Amaryllidaceae (Meerow and Snijman, 1998). This genus contains about 80 to 100 wild species that originate from southern Europe and northern Africa, with a concentration of biodiversity in the region west of the Mediterranean Sea. Different *Narcissus* species are able to hybridize, and this has led to thousands of *Narcissus* cultivars being produced for commercial use. The Royal Horticultural Society in London registers and keeps track of *Narcissus* types and all cultivars are listed in the Classified List of Daffodil Names and the International Daffodil Checklist (Kington, 2008). In the Netherlands the *Koninklijke Algemeene Vereniging voor Bloembollencultuur* (K.A.V.B) is the organization in charge of registering *Narcissus* cultivars. Different *Narcissus* types are classified according to flower characteristics into twelve groups: Trumpet, Large Crown, Small crown, Double Narcissus, Triandrus, Cyclamineus, Jonquilla, Tazetta, Poeticus, Wild species or hybrids with wild species of variants, Split crown, and all others that do not belong to any of the previous groups. The color of *Narcissus* flowers is mostly yellow, but can vary from white to pink and reddish in some cases. A color code is also given to each cultivar based on the color of the petals, eye and other flower parts.

Narcissus species are all perennial geophytes that naturally occur in a wide range of habitats. Most members of the genus flower in spring, but some do so earlier in winter and a few flower as late as autumn (Graham and Barrett, 2004). All flowers are pollinated by insects, with the most important pollinators being bees, butterflies, flies and hawkmoths (Herrera, 1995; Graham and Barrett, 2004).

Figure 2. A field of *Narcissus* plants near Lisse, The Netherlands (left), and a single plant (right).

Cultivation of *Narcissus*

Narcissus has been cultivated in the Netherlands since the sixteenth century (Hanks, 2002). Since then, cultivation has also become important in other parts of Western Europe and *Narcissus* is now one of the major ornamental spring-flowering bulb crops grown in temperate regions. It is grown commercially for the bulbs and flowers, and is also very popular in gardens, parks and other public spaces.

In terms of areas of field-grown *Narcissus* plants, the highest production occurs in the Netherlands and the UK (Hanks, 2002). Other areas where a significant amount is grown are in the Unites States, Australia and British Columbia in Canada. Israel is the main producer of the small *Narcissus tazetta* bulbs. Annual output of *Narcissus* bulbs is estimated from the area lifted per year, the average planting density, and the average weight increase from planting to lifting. What remains after the weight required for re-planting is subtracted from the total weight of bulbs is the disposable yield. The annual disposable output for the UK and the Netherlands is estimated at about 30 000 tonnes (Hanks, 2002). A rough estimation typically gives 20 000 bulbs of medium size per ton. The bulbs are sold as planting stocks, for commercial bulb forcing (making plants produce flowers in greenhouse conditions at times when flowering does not occur naturally for cut flowers and pot plants), for amenity or landscape use, and for sale to garden centers and other outlets or for mail orders. Bulbs are also exported from the UK and the Netherlands, with the main destinations being Germany, North America and France for both countries. There is some trade between the UK and the Netherlands, with bulbs of mainstream cultivars being imported in bulk to the Netherlands, while smaller amounts of more specialized cultivars are imported to the UK. The Netherlands also imports bulbs of *Narcissus tazetta* from Israel. Not only bulbs, but also cut flowers are exported from the UK and the Netherlands on a large scale. Another important export from the Netherlands is dwarf *Narcissus* grown in pots (Heinrichs, 1999). In the growing season of 2008/2009 581 cultivars of *Narcissus* were grown in the Netherlands (PT/BKD, 1999). The top five cultivars were "Tête-à-Tête", "Carlton", "Dutch Master", "Bridal Crown" and "Ice Follies", with Tête-à-Tête accounting for 38% of the total area grown (PT/BKD, 2009).

As mentioned before, *Narcissus* has been grown in the Netherlands for several centuries. Traditionally cultivation occurred in the bulb-growing region, or *Bloembollenstreek* in the south- western parts of the country, with the town Lisse as the center. This region has sandy soil and a controlled water table, and has long been regarded as an ideal location to produce the plants (Hanks, 2002). In the last 50 years *Narcissus* cultivation has also spread to more northern locations, for example de Noord, where the soil is sandy, and also more eastwards in the country. About 1570 hectares were used for *Narcissus* cultivation in 2009 (PT/BKD, 2009), making it the third most cultivated bulb plant. The total amount of hectares grown has increased in the last few decades, but the number of growers has decreased in the same time period, thus the area grown per grower has also increased. This trend of increasing scale is mostly ascribed to increases in mechanization of farming and improvements in expertise.

***Narcissus* Propagation**

Cultivating *Narcissus* from seed is a slow process, which takes three to six years from seed to flower. This is too long to be an efficient propagation method for large-scale commercial purposes, and is only used in breeding programs to obtain new cultivars. Vegetative propagation of *Narcissus* is also relatively slow, and on average 1.6-fold multiplication of bulb units per year occurs. This means that 1 bulb can give rise to 1000 bulbs in about 16 years (Rees, 1969). The multiplication rate depends to a large extent on the cultivar, and factors that further affect this include growing conditions such as planting density. Lower density gives better growth and development and also more buds that grow into offsets in the same season. In the next season the new offsets will grow and come loose or be broken off. Methods such as double scaling and chipping involve dividing the bulb into pieces or segments before planting. In this way several new bulbs can develop from one original bulb.

Micropropagation, the use of single cells or groups of cells to give rise to new plants is an effective way of increasing multiplication rates. In plants usually propagated vegetatively (like *Narcissus*) micropropagation may also be the only way to obtain virus-free plant material (Sochacki and Orlikowska, 2005). Various methods and techniques have been developed for the micropropagation of *Narcissus* (Seabrook et al., 1976; Hussey, 1982; Squires and Langton, 1990). In these methods, plants were regenerated from leaf, stem, scale and stem explants, as well as tissue from the ovary wall. A drawback of using underground plant parts as explant is the higher frequency of infection with soil microbes (Seabrook, 1990).

Some methods give rise to rooted shoots that can be planted, while others produce bulblets. The bulblets have been found to survive and grow better than the shoots when planted (Seabrook, 1990). Higher sugar concentrations in the medium can increase the amount of bulblets forming in vitro (Chow et al., 1992). Not just the amount of bulblets formed, but also the size of bulblets are important, as it is related to survival upon transplanting as well as flowering (Squires et al., 1991). Different types and concentrations of sugars, as well as the osmolarity of the medium can affect the development of the bulblets in different ways (Staikidou et al., 2005). Usually bulbs formed in in vitro propagation only produce flowers after several years (Hanks, 1993). Santos' group (Santos et al., 1998) managed to produce *Narcissus* bulblets that flowered within the first year, after about 9 months of growth. Somatic embryogenesis has also been performed with *Narcissus* of various species and cultivars, using explants from various tissue types (Selles et al., 1999; Sage et al., 2000). Malik (2008) investigated micropropagation of *Narcissus* by somatic embryogenesis from ovary explants, and looked at differences between using liquid, solid or solid-liquid media. The highest biomass increase and highest number of embryos obtained directly from the explant was with an 8-week culturing system on liquid media containing growth regulators 2,4-D and BA. The results of this study also suggest good potential for liquid media in *Narcissus* for micropropagation. At present propogation of *Narcissus* in this way is feasible only at small scale, but micropropagation of *Narcissus* continues to be the focus of many investigations.

Pests and diseases of *Narcissus*:

Nematodes

The cultivation of *Narcissus* involves, like any other commercial crop, the combating of pests and diseases. The sandy soil of the *Bloembollenstreek* allows for easy spreading of nematodes. In Narcissus, *Ditylenchus dipsaci* and *Aphelenchoides subtenuis* often affect the bulbs. In the cultivated variety Carlton, other common nematode pests include *Pratylenchis penetrans* and several *Trichodorus* and *Paratrichodorus* species. The presence of nematode infestation can be seen by various symptoms. In *D. dipsaci* for example, a section through the bulb reveals dark rings as some scales turn brown upon infestation. The bulbs may also feel soft and granulose. Darkened tissue can also be seen in *A. subtenuis* infestation, especially the skins and outer scales. Another common occurrence with this nematode is the dying off of the roots, and the presence of corky tissue where the roots used to be and around the bulb base. For ornamental flower bulb purposes, infestation by nematodes can have consequences on the quality of the bulbs, and can lead to great losses or complete failure of host crops in some cases. Attack by nematodes can affect the growth of bulbs, and make them more susceptible to fungal infection and attack by the small narcissus fly. Nematodes are also vectors for certain viruses, for example Tobacco rattle virus is spread by *Trichodorus* species. Nematodes can move from diseased bulbs through the soil to healthy bulbs. The speed of movement depends on the type of soil, and is faster in sandy soils. If no host plants are in the soil the numbers of nematode rapidly decline, but usually some remain in the soil for some time. *D. dipsaci* can remain in the soil for six to sixteen years, while *A. sebtenuis* can do so for at most two years after host plants are removed.

Two strategies are used to combat nematodes through soil treatment (Rouwette et al., 2002). The first involves the use of chemical treatments. Deep-penetrating sterilizing agents such as Metam-sodium (sodium salt of methyldithiocarbamate) is a widely used agent in bulb crops, and can be used against nematodes such as *D. dipsaci* and *Trichodorus spp*. that can occur relatively deep in the soil. It may be used once every five years or more often when *D. dipsaci* has been detected (*D. dipsaci* is a quarantine organism in bulbs). Basamid strooimiddel (scattered on the ground and worked into the soil) is used together with Metam-sodium against *D. dipsaci*. This soil treatment does not work below 40 cm below the surface.

The second strategy is the practice of inundation. This involves covering the field in a layer of water for up to 10 weeks to kill harmful organisms by oxygen deprivation. This is usually done in July or August when soil temperatures are around 17 ºC. This method is effective to significantly reduce the numbers of species such as *Pratylenchus*, *Ditylechus* and *A. subtenuis*, but it works less well for *Trichodorus* nematodes. It also works against certain fungi and weeds. Since no chemicals are used, this method is

gaining popularity, and is likely to become more important as more chemicals are being banned and as resistance to allowed ones develop.
Another important method for combating nematodes is treatment of the bulbs before planting and/or after lifting with warm water. The so-called hot water treatment (HWT) against nematodes involves heating bulbs in a large water bath for four hours at 47 ºC. In the past formaldehyde was added to the water to prevent spreading of *Fusarium* and kill free-swimming nematodes. The use of formaldehyde has recently been banned due to health concerns.

Fungi

The humid conditions in the Netherlands are favorable for fungal infections of crops. Many fungal infections occur in *Narcissus*, with different varieties and species not equally susceptible to different diseases. In general fungal infections become problematic in moist conditions and warm soil and air temperatures. Cooler temperatures tend to slow or prevent fungal growth. During storage, transport and all other handling of bulbs infections can also spread easily. Fungal infections can have serious consequences on plant quality and bulb yield, and can lead to large losses in the bulb industry. These types of diseases are often latent for a time without displaying symptoms, which can make combating them more difficult.

The most common fungal disease affecting *Narcissus* is basal rot ("bolrot"), caused by *Fusarium oxysporum*. Infection starts at the bottom of the bulb and spreads upwards to the scales. Infection can also take place in wounds at all part of the bulbs. Important place is the part at the base between mother- and daughter bulb. The infected tissue becomes discolored and soft and upon drying the bulb becomes brittle. A characteristic pinkish growth of the fungus is often seen. Diseased bulbs give rise to short, twisted plants that die early. Neck rot (koprot) is also caused by *F.oxysporium*, alone or together with several other fungi. This disease has similar symptoms to basal rot except that it starts at the bulb neck and spreads down into the bulb. Infected bulbs also give rise to weak plants or no plants emerge (Hanks and Carder, 2003; Vreeburg, 2005). To prevent *Fusarium* a number of measures have to be taken. These include: A crop rotation of at most once every four years so that infection of soil is minimized, planting late so that soil temperature is less than 12°C, planting deep enough, avoiding too much nitrogen fertilization on the field, removal of diseased plants, lifting bulbs directly after harvesting foliage, drying rapidly after lifting, not washing with water after lifting, dry storage, adding fungicide while pre-soaking and to HWT, drying well after breaking bulbs, avoiding damage as much as possible and selecting and sterlizing/treating bulbs as shortly before planting as possible. For basal rot and neck rot, as for most fungal diseases, disinfection during HWT or disinfection before planting is an essential part of control measures. The standard HWT conditions are used (2 hours at 45 ºC) and antifungal agents are added to the water bath. Alternatively bulbs can be dipped in the antifungal agents for 15 minutes in cold water.

Mechanical damage of bulbs should be avoided as much as possible, as it makes them more prone to fungal infections. Soft rot is caused by *Rhizopus arrhizus* and infection of damaged bulbs occurs at high temperatures usually during drying, at the beginning of the storage and transport of bulbs. Usually gray-brown areas develop around wound sites which turn into a soft mushy mass. Eventually a gray cobweb-like mold grows over the infected bulbs. Lowering the temperature can stop progression of the disease. Penicillium rot, as the name suggests is caused by *Penicillium* species. Disease-damaged tissue becomes brown and rotten, and this spreads to other parts of the same scale. A blue-gray to green mold is visible in infected bulbs. If the disease reaches the bulb base it may spread to other scales and spread to the whole bulb. Drying well after each damaging and disinfection HWT is done as a preventive measure, and keeping bulbs dry and warm after lifting can also prevent the development of this disease. A short period of 30°C shortly after lifting is best to prevent Penicillium rot.

Another soil fungus that may be problematic for *Narcissus* bulbs is *Sclerotinia bulborum,* which causes Black slime disease. Symptoms of the disease become visible after flowering, when the underground leaf parts become gray-brown and soft. The aboveground plant parts die down and become blackish. Bulbs can also be affected in which case the outer scale and neck turns brown and in severe cases the whole bulb disintegrates. Treatment of infected soil is necessary to prevent this disease, and is done by inundation and deep-ploughing. For bulbs a preventative measure is HWT with the fungicides, and if Black slime occurred in the field the unaffected bulbs can be given this same HWT after lifting.

For certain fungal diseases fungicide sprays are necessary to prevent severe losses or damage. Common foliar diseases of *Narcissus* include Smoulder, caused by *Botrytis narcissicola* and *Botrytis cinerea*, Fire caused by *Botrytis polyblastis* and Leaf scorch caused by *Stagonosporopsis curtisii*. These fungi produce spores that spread by wind and raindrops to aboveground foliage. Spores of *Botrytis narcissicola* and *Botrytis cinerea* can land on bulbs after lifting, and this can spread the disease to healthy bulbs. *B. polyblastis* form sclerotia on dead stems and leaves, and if in contact with the soil there germinate to produce sexual spores. Wind and rain brings these in contact with the flowers, where many asexual spores are made. This infection spreads to the stems and foliage and when the foliage dies down due to the infection, sclerotia are formed again to complete the cycle. Similarly, a heavy *Stagonosporopsis curtisii* infection can cause

the stems and leaves to die, and the fungus can survive in the dead tissue that remains behind in the field. The fungus can also come in contact with the bulbs after lifting, where it can survive in and on the bulb neck and in the bulb skins. When leaves emerge from these bulbs, they will become infected with the fungus. Specific agents are sprayed in the field for each of these diseases.
Botrytis narcissicola is responsible for another disease affecting the bulbs of Narcissus, namely type I “Vethuidigheid”. This disease gives one or more of the outer scales a fatty and glossy appearance, and they later become tough and leathery with a dark brown colour. As with “Huidziek”, bulb scales and skins become loose-fitting. This disease can also be caused by *Phoma leveilli*, in which case it is classified as type II. For both cases, a standard HWT with chemical additives as for *Fusarium* is the typical preventative measure. Root rot is caused by *Pythium* species and affects the roots of *Narcissus*. They develop rotten tips and bulb development is impaired, and sometimes the bulbs look like they are affected by Huidziek. Bulb sterilization with a standard HWT is used as a preventative measure, but this disease is more of a problem in potted *Narcissus* bulbs than with field-grown plants.

Some of the fungi that infect *Narcissus* bulbs in the Netherlands belong to genera that are known to produce mycotoxins. Mycotoxins are low-molecular weight secondary metabolites produced by various fungal genera including members of *Fusarium* and *Penicillium*, that can cause disease and death in humans and other vertebrates (Bennett and Klich, 2003). *Fusarium* mainly affects plants while growing in the field as a plant pathogen, and produces mycotoxins just before or after harvest, while *Penicillium* is more common as a contaminant in stages after harvest, for example during drying or storage. Some important mycotoxins produced by *Penicillium* include citrinin and patulin, while *Fusarium* are known to produce fuminosins, trichothecenes and zearalenone. Biosynthesis of mycotoxins is determined by a wide array of physical, biological and chemical factors. Time, temperature, humidity and physical damage by other organisms such as insects interact in complex ways to induce mycotoxin synthesis (D’Mello et al., 1998). Since *Narcissus* has not traditionally been cultivated for consumption or pharmaceutical use, the presence of mycotoxins has up to now not been of much importance. It is not known whether infection with *Penicillium* or *Fusarium* results in mycotoxin production in the bulbs. This may need to be investigated if *Narcissus* bulbs are to be used for pharmaceutical purposes.

Insects
The main insect pest of *Narcissus* is the larva of the large Narcissus fly (*Meredon equestris F*.). The female fly lays eggs at the foot of the plant at the beginning of summer. After a week or two larvae emerge, which then move to the bottom of the bulb

where they penetrate it. The larvae live in the bulbs from the end of June and can reach lengths of up to 18mm. In spring the larvae leave the bulb to form pupae just below the surface of the ground. In May an adult fly emerges again from the pupa to complete the cycle. Bulbs infested with these larvae often appear slightly darker in color and are less well developed. The bulbs may feel corky and often a hole is visible at the bottom of the bulbs. The larvae burrow in the bulb and make small passages, which become filled with grey granular material, and have a larva still in it after lifting. The grow points of larva-infested bulbs are usually lost, so that infested single-neck bulbs will not produce plants. Double-necked plants may emerge but generally very weak plants are formed.
A standard HWT of 2 hours at 45 ºC is given as a preventative measure against narcissus fly. An additional measure has been investigated lately, namely dipping bulbs in 0.05% Admire (imidacloprid) for 15 minutes just before planting, and seemed promising against a new attack in early summer (Conijn and Korsuize, 2007). The standard HWT is also quite effective in preventing attack from other insect pests of *Narcissus* such as Narcissus mite. Slugs can attack *Narcissus* plants, but are not a very big problem and usually no action is taken against them. The small Narcissus fly is often seen during storage when bulbs are infected by *D. dipsaci*. Only diseased bulbs are infected and destroyed by the larvae of this small fly.

Viruses

Most *Narcissus* bulbs are infected with one or more viruses at any given time (Mowat, 1980). At least 24 viruses have been reported to infect *Narcissus*, but not all are commonly found (Brunt, 1995; Asjes, 1996). The presence of viruses affects the health of the plant, and can result in bulb yield losses of up to 30% (Asjes, 1996). Obtaining virus-free bulbs is difficult, and is usually only possible through micropropogation. Depending on the combination of viruses present, a variety of disorders can result, the names of which usually indicate the most dominant symptom (e.g. “Streperigheid”, “Blad bruin”, “Topvergeling”). A number of factors can affect the diagnosis of a viral disease, as certain conditions will cause some symptoms to be more pronounced. For example, long periods of cold temperatures during the growing season often cause more obvious symptoms than with higher temperatures. Other factors that can affect the degree to which viruses cause symptoms are the developmental phase of the plant, resistance of a particular cultivar, growing conditions such as soil parameters and plant density. Plants can also be infected with one or more viruses without showing symptoms at all.
Clearly the virus situation in *Narcissus* is complicated. Not much can be done to combat viruses once plants are infected, but some measures can be taken to prevent them from spreading. Growers are usually on the lookout for viral symptoms and remove seriously affected plants from the field. The symptoms for viral diseases mostly affect the leaves

and stems.

Physiological and other disorders of *Narcissus*:
Apart from attack by insects or infection by pathogens, other factors may also cause plants to be damaged. These can cause poorer quality bulbs and flowers, or complete loss of crops. Abiotic factors related to weather conditions may cause disorders, as well as treatments performed by growers during cultivation. These will be briefly discussed below.

Frost Damage:
Different *Narcissus* cultivars are not equally sensitive to frost damage, but for most types soil temperatures below -2 ºC are harmful if it continues for a few days. Light frost damage causes shorter plants and twisted leaves, with the leaves often being yellow-green with a rough appearance. The stems can become hollow and break easily. Starting from the border with the scales, the bulb base becomes glassy and gray, with a darker color if the damage is more severe. With light frost damage plants are likely to die early, and with more heavy damage bulbs will not produce plants at all. Long lasting frost can also cause a frozen ice layer in the soil that interferes with water drainage and can cause water-logging and lack of oxygen. Frost damage also makes bulbs more prone to fungal infections. To prevent heat loss from the ground a straw layer is often placed on the field after planting bulbs, or a cover crop such as wheat is planted.

Water logging:
Too much water in the soil causes roots to become rotten starting from the tips. The plants that emerge grow less well, have patches or yellowish horizontal bands on the stems and may not flower. In severe cases the roots rot away completely, and no plant is produced. The bulb tissue can become dark gray to black, but usually the bulb itself is not affected too much. Water-logging can be avoided by ensuring a good structure and drainage of the soil.

Sun scorch:
If bulbs are exposed to strong sunlight on the day of lifting or shortly after, the tissue may be damaged. When this happens the bulb tissue becomes grey-white in color, dries out and becomes very hard. The damage may go as deep as three scale layers, but may be hidden by brown bulb skins. Even if the bulbs do not seem to be damaged, plants grown from those with light sun scorch damage may show some effects. Sun scorch damage can be prevented by not lifting bulbs on very sunny days, or protecting lifted bulbs from strong direct sunlight.

Mechanical damage:
Mechanical damage of the bulbs can occur when they fall or are squashed. Damaged parts have brownish patches on the skins and under these dark gray patches ("stootplekjes") develop. The discoloration may go as deep as four scale layers. Bulbs are most vulnerable to this kind of damage during or just after lifting. The discoloration, though unsightly, usually does not affect the quality of the bulbs for flower production, although it does increase the risk of a *Botrytis* or *Penicillium* infection developing. For this reason mechanical damage should be avoided when handling bulbs by for example minimizing the falling distance in mechanized processes.

Chemical treatments:
Excessive use of herbicides in the field can have a negative effect on plants. If plants have already emerged from the bulbs, herbicides may cause gray-white, papery patches on the leaves. This damage seems to affect the bulbs as well as the aboveground parts as damage in one growing season can lead to bulbs forming plants with yellowish leaves the following year. Very heavily damaged bulbs will not produce plants the following year, or those that do emerge will die prematurely. It is not well known whether pesticides have a negative effect on the plants or bulbs.

Hot water treatment damage (*kookbeschadiging*):
HWT can cause some damage if the difference between the storage temperatures before the treatment is much lower than that of the water bath. When this happens, the tips of the roots and the tissue between the bulb base and scales become gray or black. Parts of the scales may also become gray in cases of severe damage. Plants emerging from damaged bulbs will show some effects, such as yellow patches near the tip, skew-growing leaves and dried out flower bud. In very severe cases the flower bud and stem dries out completely while still in the bulb. If a HWT is to be performed at 47 ºC against nematodes, a warm pre-treatment for one week at 30 ºC helps to reduce the damage. For the standard HWT (2 hr at 45 ºC) against nematodes, mites and flies a warm treatment is not required unless the bulbs were stored at temperatures below 20 ºC.

Quick overview of chain: how it is typically done for flower bulb production
Narcissus bulbs are planted in late summer or autumn, after which root growth from the bulb occurs rapidly. The shoot grows inside the bulbs but eventually this is slowed down by decreasing temperatures as winter approaches. Most *Narcissus* bulbs require a cold period in winter which is necessary for rapid, synchronous shoot growth and anthesis once it gets warmer (Hanks, 2002). Shoot growth is then determined by ambient temperatures and bulbs grow rapidly from around the time of anthesis. In summer leaves senesce and as a result bulb growth also slows or stops. Flowers are

harvested in spring, and the bulbs are lifted in summer. In the Netherlands an one-year cultivation system (one-year down) is used as opposed to 2 year (two-year down) system. This means that bulbs are lifted after each year, as opposed to only after two growing seasons. Two-year systems are employed mostly in non-sandy soil types, where slower growth and more difficult and expensive lifting and harvesting procedures make it more feasible to adopt a two-year system. Two year systems have some disadvantages mostly related to pests and diseases, so in the Netherlands sandy soils and ease of mechanization makes it more favorable to lift bulbs every year. A brief overview of the steps in the production chain for *Narcissus* bulbs is given as it is done for flower bulb production.

Planting stock:
The production chain starts with the planting stock. The choice of planting stock depends on the desired characteristics of the harvested bulb. Material can be obtained from auctions or private sales, or grown under contract arrangements, or from a farm's own planting stock. Bulbs are available in different grades, based on their circumference. More details on sorting and grading bulbs will be discussed later.

Bulb treatment before planting:
A standard part of the production chain is treatment of the bulbs before planting. This involves a “hot water treatment” (HWT) during which bulbs are heated in a water bath. The standard HWT involves heating bulbs at 45 ºC for 2 hours, and this is a good preventative measure against nematodes, mites and narcissus flies. Agents are added to the water bath, to help prevent certain fungal infections. This standard HWT is always done in August or September before planting in September or October. If bulbs are infected with nematodes, a longer and warmer HWT is given (47 ºC for 4 hours) and the bulbs are presoaked before the treatment. Forced bulbs always get a 4 hour treatment at 47 °C.

Soil before planting:
Before bulbs are planted, the soil should be prepared. This involves processes to ensure a good soil structure (good ratio of air, organic material, water) adequate drainage and nutrient levels. All the soil in the Netherlands is good for *Narcissus* growth and suitable for mechanization. Nematodes are however a bigger problem when different bulbs are grown every year on the same fields. Soil sterilization is sometimes part of the procedure to prepare soil for planting. This may involve chemical means or alternative methods such as inundation. Crop rotation is usually practiced in flower bulb production and usually *Narcissus* is grown on the same site once every four years.

Planting:
Narcissus bulbs are usually planted in autumn, around the end of September and in October. The advised depth of planting is 10-15 cm below the surface, and planting density depends on the type of bulbs planted. An average planting density is about 25 ton per hectare (for varieties like Carlton). When planting on sandy soil, usually bulbs are planted in beddings, in rows or covering the entire field. In heavier soils usually bulbs are plants in raised ridges.

In-field treatment:
Since *Narcissus* is prone to frost damage during the winter months, a common practice is to cover the planted bulbs with straw to keep soil temperatures from dropping too low. Sometimes a cover crop (rye, wheat) is also grown over the bulbs to achieve the same affect. Cover crops are often removed by spraying with an herbicide before the *Narcissus* emerges. Herbicide treatments are commonly employed against weeds, and are typically applied a few times during the season. Further chemical treatments in the field include pesticide sprays, especially fungicide and sometimes insecticides. This can be done preventively or diagnostically depending on the grower. Irrigation is also part of the field operations, and depends on the weather conditions during the growing season. Flower harvesting occurs in springtime.

Bulb lifting:
Bulbs are lifted from the ground in mid-July to beginning of August. Shortly before lifting the foliage is removed. Bulbs are lifted with a lifting-machine and then left in the field to dry out for a few days. Once dry enough the bulbs are picked up into bulk bins of 600 – 1200 liters, and then moved to sheds for further treatment.

Bulb handling for sale/replanting:
After lifting and drying, the bulbs are cleaned using a shaking sieve to remove soil particles. They are then transferred to a sorting machine for sorting and grading. Varieties with rough bulbs such as Carlton are sorted with spijlenplaten (plates with oblong holes) and often double-nosed bulbs are separated from round bulbs before sale. Often the stock to be sold consists of offsets and mother bulbs. Sometimes bulbs are sold per kilogram and are then not sorted. Round-holed plates are used to sort *Narcissus* with smaller bulbs such as Tête-à-Tête. Unusual or defective bulbs are removed from the bulk stock. Bulbs to be sold (“leverbaar” stock) are typically stored at about 20 ºC, at a relative humidity of 60-70% after lifting and drying. Bulbs to be planted always get a HWT. *Fusarium* infection takes time to become visible, so it is better to leave the selection of bulbs until as late as possible.