Factors affecting galanthamine production in Narcissus

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Chapter II

Factors Affecting the Production of Galanthamine and Other Metabolites in *Narcissus* plants

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Abstract

Narcissus is one of the most well-known ornamental plants with quite a high value. Moreover, it is also the source of several compounds which have demonstrated several biological activities. These bioactive compounds are of great interest as medicines in different forms and combinations. Galanthamine is one of these bioactive compounds which is quite well known. Galanthamine is an acetylcholinesterase inhibiting compound which is marketed in form of medicine for Alzheimer's Disease (AD). Galanthamine can be produced in two ways i.e. natural extraction and synthetic production. *Narcissus* bulbs are considered one of the most important natural sources of galanthamine due to its metabolite contents and bulb production at large scale. Galanthamine can be found in all parts of the *Narcissus* plant in varying amounts at different stages of plant growth cycle. Many aspects of galanthamine production have been studied by various scientists over the time. However, there are still many aspects of galanthamine accumulation in *Narcissus* which are not fully known. This review provides an organized and critical overview of the localization and biosynthesis of galanthamine, the effect of different agricultural practices, environmental factors, pests and diseases on the overall production of *Narcissus*. Moreover, it also provides an overview of how these factors can affect the galanthamine and other metabolites production in *Narcissus*.

Keywords: *Narcissus* Interaction; Biosynthesis; Diseases; plant density.

Introduction

Flowering bulbs are also defined as ornamental geophytes. These bulbs exhibit great diversity in their morphology, growth and developmental biology [1]. This diversity is abundantly clear in physiological responses to different environmental factors which initiate/enhance the production of secondary metabolites. Among these secondary compounds, a wide range of compounds is biologically active which are important to treat various diseases [2-4]. Horticulturally speaking, these flowering bulbs contribute significantly to the global floriculture industry where they are utilized for the commercial bulb and flower production. The flower production part includes in-season outdoor flower production and forced fresh-cut flowers from potted plants at other times of the year. These flowering bulbs are also used for landscaping which includes private gardening at small scale.

Although ornamental geophytes belong to more than 800 different genera, but the flowering bulb industry is dominated by seven genera: *Tulipa, Lilium, Narcissus, Gladiolus, Hyacinthus, Crocus,* and *Iris*. From all these genera, *Narcissus* is very important due to its medicinal value in addition to flower and bulb production [1, 5]. *Narcissus* possesses chemicals/compounds with antiviral and antitumor properties as well as an anticholinesterase activity. The alkaloid galanthamine (GAL) is one of the most studied compounds in the field of anticholinesterase activity [6, 7].

Galanthamine is an isoquinoline alkaloid. It inhibits the enzyme acetylcholinesterase (AChE) in a competitive way on the enzyme and the action is reversible. That is the reason it is an important therapeutic agent for Alzheimer's disease (AD) [8]. Alzheimer's is marked as a type of dementia. AD is a complex, neurodegenerative, multifactorial and progressive disease which mainly affects the elderly population. It is estimated to account for 50–60% of dementia cases in persons which are 60 years or older. The characteristic symptoms of this disease are a loss of memory as well as impairment of multiple cognitive and emotional functions which are required on a daily basis [9].

Evans *et al.,*[10] have reported that the 4th leading cause of death in the elderly people is Alzheimer's disease and the occurrence of this devastating illness is increasing day by day. Until the end of 2008, there were about 30 million people, which were suffering from dementia worldwide. This figure is expected to rise to over 100 million till 2050. More than 60% of people with dementia belong to the developing countries, which will rise to 71% at the end of 2040. The rapid growth of this disease is occurring mostly in the elderly population of China, India and their south Asian and western Pacific neighbors. According to a recently published report, the number of AD patients in the USA alone is approximately 5.4 million, which is expected to rise to 16 million in 2050. Only in USA about 200 billion US dollars are spent on the treatment and care of dementia on a yearly basis [11]. At the same time, the number of AD patients in the UK is around 800,000 which are expected to rise to 1 million by 2021. Only in 2012 about 23 billion British pounds were spent to provide care and medicine to these AD patients. It was also stated in the report that women and less educated people are the most affected people by this disease.

Galanthamine is an important agent to treat the symptoms and slow down the progression of this disease in the patients and improve their quality of life. Johnson and Johnson's pharmaceuticals had both a patent for galanthamine for the treatment of AD as well as for the synthetic production of galanthamine. But quite some galanthamine is produced by extraction of Narcissus bulbs.

Other than galanthamine there is a long list of compounds which can be/are extracted from the *Narcissus* and demonstrate many potentially beneficial effects for human beings. These compounds mostly include alkaloids, some dyes, carotenoids, lectins, and volatiles. The volatiles are used in perfumes. Some of the *Narcissus* lectins have shown activity against human immunodeficiency virus [12] in *in-vitro* studies [13, 14]. A few genes have also been isolated from *Narcissus* and used for different purposes in other crops. Some examples are shown in **Table 1.1** in the previous chapter.

The aim of this article is to provide an overview of the agricultural aspects of the industrial production of galanthamine from plants. The occurrence of galanthamine is first discussed after which the present knowledge on the biosynthesis of galanthamine and related alkaloids is reviewed. The factors that have been studied for the optimization of galanthamine production in the bulbs are discussed in detail. This includes also a review of the effects of weeds, insects and diseases on the plant biomass production and alkaloid levels in the plant.

Occurrence and Localization of Galanthamine:

Many plants of the genera *Amaryllis, Crinum, Galanthus, Haemanthus, Hippeastrum, Hymenocallis, Leucojum, Lycoris, Narcissus, Nerine, Sternbergia, Ungernia, Zephyranthes,* contain galanthamine [15]. Currently, in Europe, it is extracted from the daffodils (*Narcissus* cultivars) and summer snowflake (*Leucojum aestivum*) while in China it is extracted from red-tubed lily (*Lycoris radiata*). It is also prepared from *Ungernia victoria* in Uzbekistan and Kazakhstan. Of these plants producing galanthamine, *Narcissus* contains the 2nd highest amount of this alkaloid which is about 0.35-0.40%. this quantity is lower than the 2.5% in *L. aestivum* which is at the top spot for highest quantity. However, *Narcissus* has the advantage of faster growth of the bulbs if compared to the snowflake, making the production per hectare the highest.

Torras-Claveria et al. [16] analyzed more than 100 cultivars of *Narcissus* for the alkaloid content and acetylcholine esterase inhibition. The highest levels of galanthamine were found in the leaves of some cultivars (about 0.1- 0.15 % of DW), but most had about 0.02 %. In the bulbs, similar levels of about 0.02% galanthamine were found, with a few almost reaching the 0.1% level, with the cultivar yellow wings being the highest in both leaves (0.14%) and bulbs (0.13%) of all cultivars tested. The wild *N confusus,* known for the highest level of galanthamine in *Narcissus* plants*,* scores 0.69% of DW for the leaves and 1.32% for the bulbs. Also, the *N. hispanicus* scored highest with 0.46% for the leaves and, 0.09% for the bulbs. None of these cultivars reached the level of the commonly used variety Carlton, which has a galanthamine level of 0.19 % of DW (Kreh, 2002). Breiterova et al. [17] studied 40 taxa of *Narcissus* for their alkaloid profile and cholinesterase inhibitory activity. Based on semi-quantified data with the help of GC-MS, they concluded that the ornamental taxa *Narcissus* cv Sundisc, *N.* cv. Jenny, and *N.* cv. Sealing have higher amount of galanthamine, lycorine and haemanthamine respectively.

There seems to be other sources of galanthamine that have a higher level of this alkaloid than the Carlton variety. However, the change from Carlton to another source is not a simple process. The main issue is that the production of galanthamine in the original drug master files is defined as being extracted from the bulbs of specific Carlton variety. The use of any other variety or source for galanthamine production will require a new drug master file. This file needs to have extract's safety data sheet among other parameters. That means possible toxicological studies are required in case of different contaminations in galanthamine, e.g. other minor alkaloids. Considering the data on the various cultivars and species, it is clear that each source will bring other potential contaminations. Moreover, large scale extraction of leaves is more complex than extracting the bulbs. This hampers the possibilities of using other sources for galanthamine production.

Galanthamine can be found in all parts of the plants throughout most of the growth season. Although it is not well-known which factors are responsible for the production and accumulation of galanthamine in plants. In *Narcissus* plants the maximum amount of galanthamine is found in the bulbs. The amount of galanthamine varies in bulbs during the season. Lopez and co-workers [18] performed an experiment with *Narcissus confusus* to check the pattern of galanthamine accumulation. They found that during the season the contents of galanthamine decreased in the underground parts while they increased in the upper parts. The maximuem amount of galanthamine in the aerial parts of *N. confusus* was at the flower senescence stage while in the underground parts it was during the pre-resting period. It seems that the amount of galanthamine is reallocated during the season. It was also reported that the basal plate and inner parts of the bulb are the locations with the highest amount of galanthamine in the bulb [19]. However, there is very little information available about the localization of the galanthamine biosynthetic pathway. Lubbe et al. (2013) showed that the galanthamine levels in the various organs of *N. pseudonarcissus* cv Carlton differ over the growth cycle of the plant. Particularly haemantamine showed high levels (0.5%) in the leaves before flowering and was by far the major alkaloid even higher than the alkaloids in bulbs and roots. However, it showed a marked decrease after flowering started to end at a level similar to galanthamine. The major alkaloid galanthamine in the bulbs has highest level just before flowering (almost 0.3%) and after a slight decrease again seems to have increased again at the time of shoot senescence. The major conclusion is that harvesting following the normal procedure of bulb production is the most favorable for galanthamine production, as it will yield the highest weight of bulbs per hectare in the whole growth cycle.

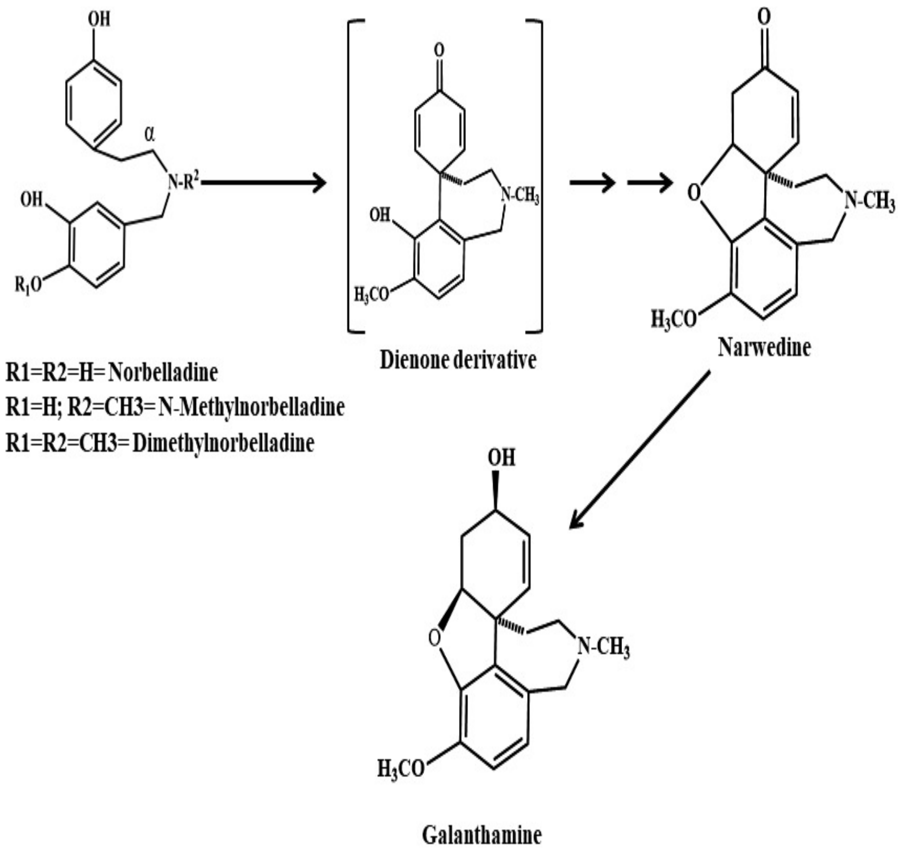

Figure 2.1. First postulated biosynthetic pathway for galanthamine by Barton and Kirby, and Barton *et al*. [20] (reviewed by Eichhorn *et al.* 1998) [21].

Biosynthesis of Galanthamine:

Although galanthamine is mostly extracted from plants, several attempts have been made for total or semi-synthetic production as well as plant biosynthesis. Some part of the galanthamine from the total available in the market is synthetically produced [22, 23]. When extracted from plant material, the costs of galanthamine are determined by the quality of plant material in terms of total amount of galanthamine produced per hectare. This generally means that a high biomass amount with high levels of galanthamine is required. Moreover, a number of other alkaloids such as narwedine and *N*-demethylgalanthamine can be extracted and purified from the same plant material. These alkaloids are thought to be the precursors for galanthamine biosynthesis in plants, thus making them compounds of interest for bioconversion to obtain more galanthamine.

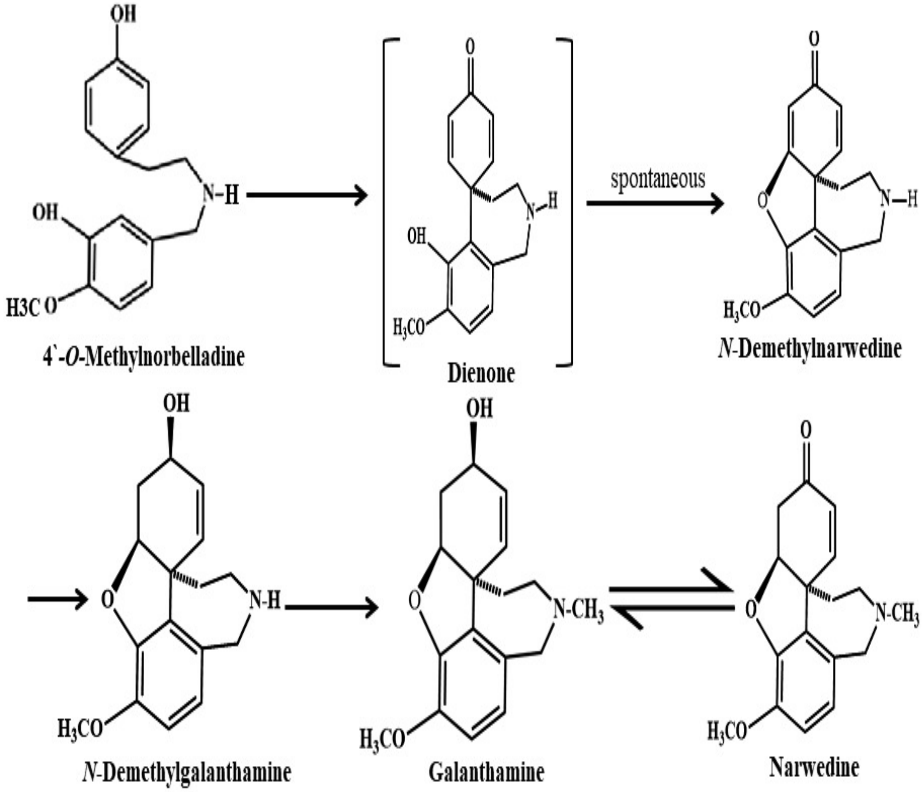

Figure 2.2. Modified postulated pathway for galanthamine biosynthesis described by Eichhorn *et al*. (1998) during their study of organs of radioactively labeled *Leucojum aestivum* plants.

On the other hand, it is also of interest to further study the biological activities of these byproducts as they possibly could add extra value to the galanthamine production. Eichhorn and co-workers [21], Berkov and co-workers [24] along with Takos and Rook [25] stated, that knowledge of biosynthesis of galanthamine can provide clues for the production of galanthamine with the help of biotechnology. Gene technology with the help of detailed information of the biosynthetic pathway would allow cloning and overexpressing different genes to produce galanthamine in other organisms, or at higher levels in a *Narcissus* or related species. Concerning the biosynthesis of galanthamine, it was postulated that norbelladine is the common

An experiment was performed in which α-^{14}C-labelled norbelladine was fed to *Narcissus pseudonarcissus* cv. King Alfred as a precursor to studying the pathway of galanthamine. The results showed that it was incorporated in the biosynthetic pathway and a biosynthetic sequence (**Figure 2.1)** was proposed [20, 26]. Fuganti [27] reported, that in *Leucojum aestivum* 4'-*O*-methylnorbelladine was incorporated into galanthamine. There was another postulation that narwedine is the precursor for galanthamine production and it was reported that [^{3}H] narwedine was incorporated into galanthamine biosynthetic pathway [22].

In 1998 Eichhorn *et al*., [21] performed an experiment to check these different postulates. They applied radioactive as well as ^{13}C-labelled 4'-*O*-methylnorbelladine and *N*-methylated 4'-*O*-methylnorbelladine to organs of field grown *Leucojum aestivum* plants. The results showed that incorporation of 4'-*O*-methylnorbelladine was 27% into galanthamine and 30% into the *N*-demethylgalanthamine. While the incorporation of *N*-methylated 4'-*O*-methylnorbelladine is about 10% in both the above-mentioned compounds. They also showed that *N*-demethylgalanthamine is *N*-methylated into galanthamine as the last step of this pathway.

Eichhorn and co-workers [21] have concluded from this experiment, that narwedine is not the direct precursor of galanthamine in the biosynthetic pathway. There is a possibility that both galanthamine and narwedine are in equilibrium explaining incorporation of narwedine into galanthamine in the previous studies. They proposed a new scheme for the biosynthetic pathway (**Figure 2.2)**. However, it could not be excluded that *N*-demethylnarwedine also via narwedine can be a precursor for the formation of galanthamine.

The early parts of the biosynthetic pathways leading to the various *Narcissus* alkaloids are shown in **Figure 2.3** though the full proof for the different galanthamine producing plant species is not yet given. This scheme was proposed by Bastida *et al.*[28].

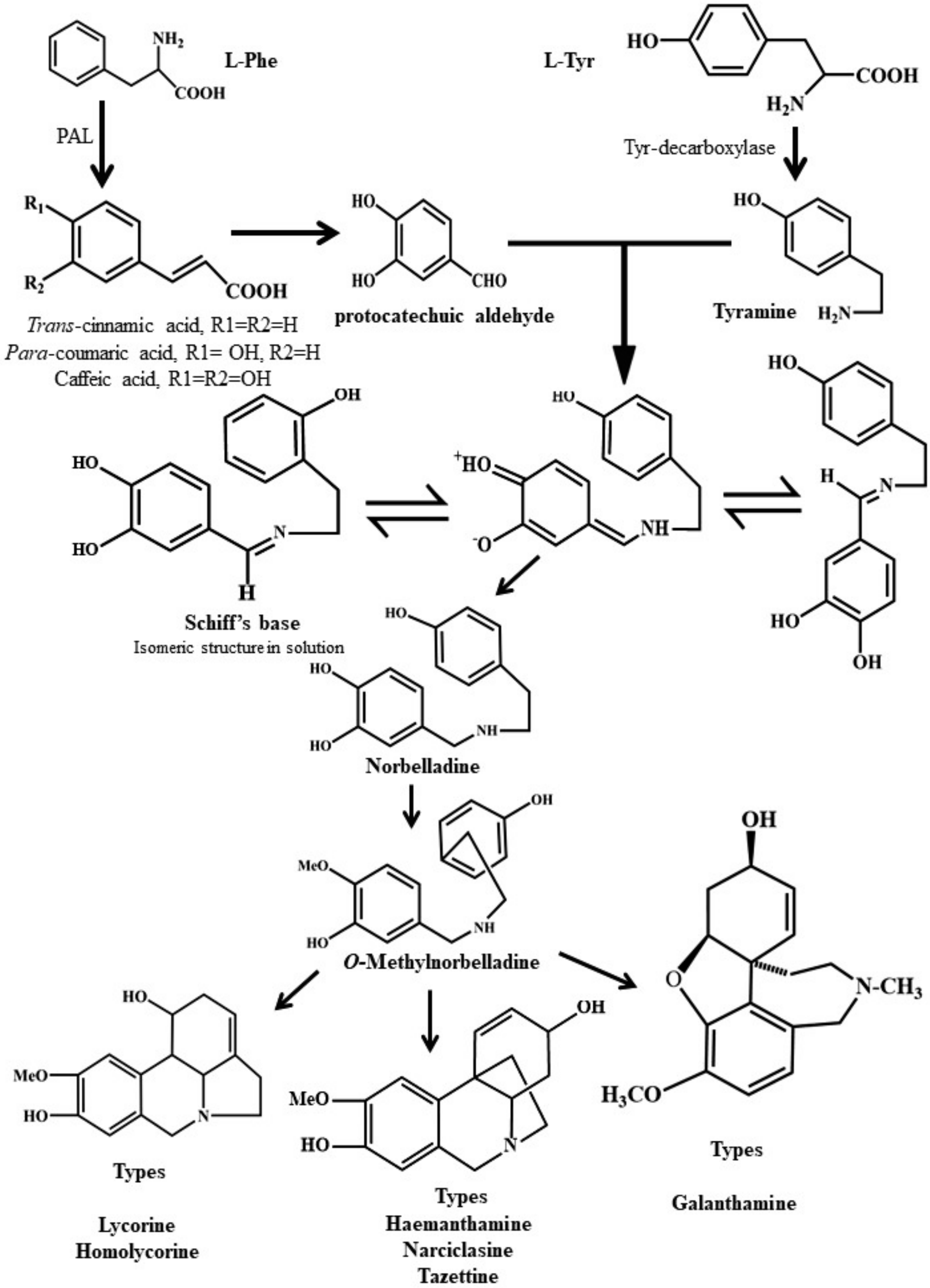

Figure 2.3. The biosynthetic pathway for galanthamine and other Amaryllidaceae alkaloids as reported by Bastida *et al.* (2006) in their review of plant sources of galanthamine.

They reported, that the biosynthetic pathway of galanthamine and similar alkaloids starts from L-phenylalanine (L-phe) and L-tyrosine (L-tyr). They have tested this assumption in *Narcissus* variety 'King Alfred' by feeding C-β L-phe. They found, that the final product in this feeding experiment was protocatechuic aldehyde or a derivative, while L-tyr was not degraded into any other product. They reported that a Schiff's base was formed in the plants from the reaction of the amine and the aldehyde. Support for this claim came from the example of *Crinum* species where two Schiff's bases have been found. Based on this experiment, they claimed their assumption was proved. They thus postulated that L-Phenylalanine (L-phe) and L-tyrosine (L-tyr) are the primary precursors, which lead into *O*-methylnorbelladine. Then from *O*-methylnorbelladine different alkaloids are formed by a different type of oxidative phenolic couplings (lycorine - *ortho-para';* narciclasine*, para-para';* and galanthamine - *para-ortho'*) [29, 30]. All assumptions for the galanthamine biosynthesis are summarized in **Figure 2.4.** *O*-methylnorbelladine is thought to be the precursor of galanthamine, but also of other groups of alkaloids in *Amaryllidaceae*species. In several studies, *Leucojum aestivum* was used as a plant source, whereas Barton and Kerby (1962) and Barton *et al.,* (1963) used *Narcissus* as plant material. The use of different model plants might explain that different precursors were found to be involved, but this requires further studies. Eichhorn *et al.*[21] reported that ovary walls and flower stalks of *Leucojum aestivum* are the best organs for precursor incorporation in biosynthesis experiments. They found that all the other organs except petals and fruit walls did not show much alkaloid metabolism, so they are not suited for incorporation studies. By feeding 4 '-O-Methylnorbelladine *Leucojum aestivum* L. shoot cultures showed an increase in galanthamine and lycorine contents [31].

The enzyme Norbelladine 4 '-O-Methyltransferase was identified and the gene was cloned from *Narcissus sp aff. pseudonarcissus* [32, 33] and its activity was confirmed by expression in *E. coli.*. The same group identified CYP96T1 in the transcriptome of some Amaryllidaceae species. This cytochrome P450 encoding gene shows the same expression pattern as Norbelladine 4 '-O-methyltransferase in *N. sp aff. pseudonarcissus* and was subsequently sequenced. The enzyme seems to be involved in the oxidative phenol coupling that leads to the basic structures of the AA [29]. This CYP enzyme has as main product the *para-para'* coupling product, i.e. the pathway leading to among others haemantamine. The reaction gives equal amounts of the two possible enantiomeres at C-4 as the major compounds. Less than 1% of the reaction product was the *para-ortho'* coupling product N-demethylnarwedine, the immediate precursor for galanthamine. No *ortho-para*' coupling product was observed.

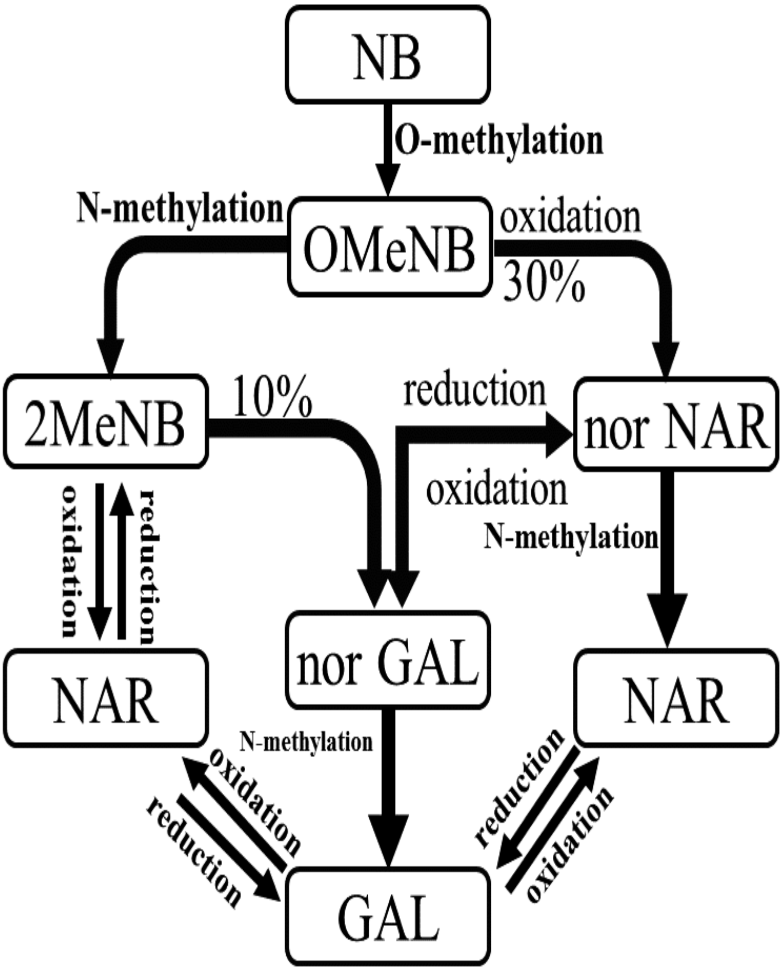

Figure 2.4: Summary of the different biosynthetic steps in the conversion of norbelladine into galanthamine as described in various reports. NB= Norbelladine, 2MeNB= N- methylated, OMeNB= O-methylnorbelladine, nor NAR= norNarwedine, norGAL= norgalanthamine, GAL= Galanthamine, NAR= Narwedin

[30] presented a comprehensive metabolome and transcriptome study from *N. pseudonarcissus* 'King Alfred' providing an important basis for future studies on the

biosynthesis. Among others two tyrosine decarboxylase genes two phenylalanine ammonia lyase genes were found in the transcriptome, that might catalyze reactions in the early parts of the galanthamine pathway. The first result of this transcriptome analysis was the cloning of the gene encoding the enzyme that catalyzes the first committed reaction in the galanthamine biosynthesis, norbelladine synthase [34]. Amaryllidaceae alkaloids share a common precursor, norbelladine, synthesized by an enzyme catalyzing a Mannich reaction involving the condensation of tyramine and 3,4-dihydroxybenzaldehyde. They described that this novel enzyme helps in catalyzing the first committed step of AA biosynthesis, which will facilitate the establishment of metabolic engineering and synthetic biology platforms to produce AAs.

With several genes of the pathway cloned, the next step comes in sight, the expression of these genes in a suitable host. As it concerns only a few genes these could be transformed into a microorganism like *E. coli*, a yeast or another plant species that is easy to grow on large scale. Though stereospecificity of the '*para-ortho'* coupling needs to be achieved.

Biotransformation can be another way to produce galanthamine at low cost. For this purpose, there were attempts made for the microbial transformation of galanthamine precursors. Spassov et al. (Spassov et al., 1986) tested a number of microbial strains to transform a belladine derivative into galanthamine but were not successful. No data was found reporting the efforts to isolate enzymes/genes encoding the different stages of galanthamine biosynthesis. With no success in biotransformation and biosynthesis, the focus of research changed towards the chemical synthesis of galanthamine.

Total synthesis of galanthamine is quite feasible and it was described in many previous studies. Czollner *et al.* [35] developed a complete (9 step) synthesis method for the production of (-) galanthamine which can provide an overall yield of 18-21%. The yield of synthetic galanthamine was improved by Node *et al.* [36] via improving the pivotal phenolic oxidative coupling reaction of norbelladine-type derivatives. Marco-Contelles *et al.* [37] have reviewed most of the synthetic approaches and patents in this field. Nugent and co-workers (Nugent et al. reported novel approaches to the synthesis of galanthamine (Nugent et al. 2015, 2016).

Factors Effecting Bulb Production:

Different factors affect the bulb yield in the field. Le Nard and De Hertogh [38] have pointed out a number of factors, which affect the bulb yield in general (**Figure 2.5**). Although these factors affect the plant independently, the interaction of these factors is also worth mentioning. Thus, it is important to find the best combinations of different factors for better bulb production as well as galanthamine production from plants.

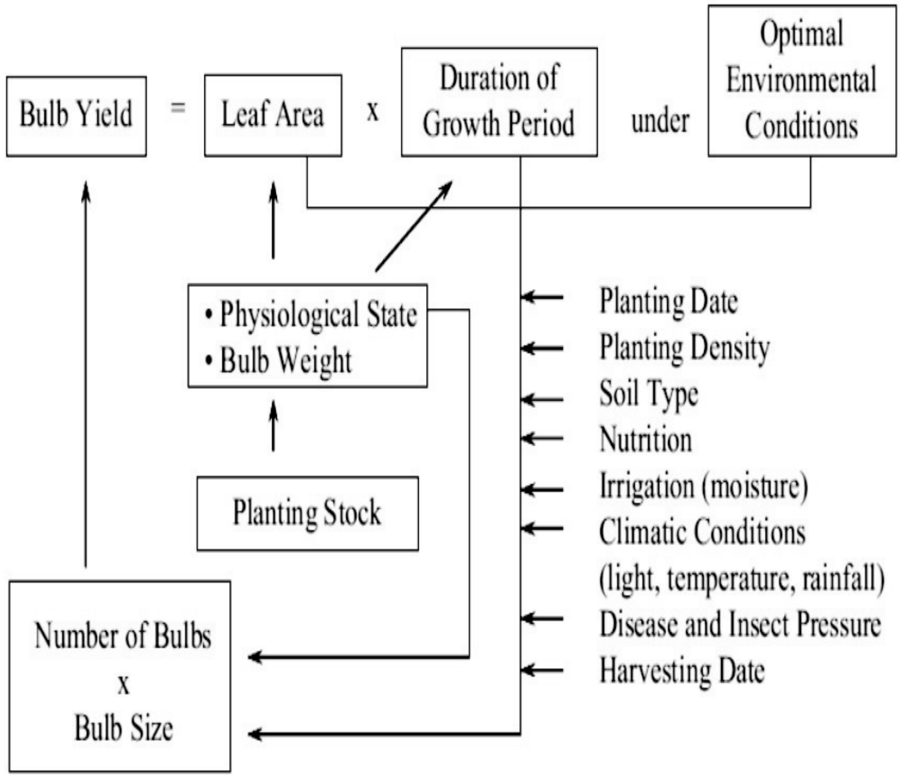

Figure 2.5: Major factors affecting plant growth and bulb production of ornamental geophytes as reported by Benschop *et al*. [39] in their book about the bulb production of ornamental plants.

For *Narcissus,* there is a reasonable amount of data available on the cultivation for ornamental purposes but very little information on the effect of cultivation practices on the plant metabolome and alkaloid production.

The bulb growers are familiar with the effect of fertilizers on the growth of *Narcissus* plants. Phosphorus in the form of fertilizers leads to good root development and strong plants, potassium fertilizers are of great importance to produce strong bulbs and good flowers while nitrogen fertilizers help to grow a good number of leaves and strong bulb development after leaf senescence. However, excessive amounts of nitrogen lead to the formation of soft bulbs in *Narcissus* plants. These soft bulbs are very sensitive to plant diseases and often rot [40].

The use of either nitrogen or potassium/magnesium (N, P, K) fertilizer increased the galanthamine contents of the bulbs and leaves of *Narcissus* plants significantly over the control. An increase in galanthamine content by 70% was achieved in the case of nitrogen fertilizer, and of 113% after using potassium/magnesium fertilizer. The results clearly demonstrated the effect of fertilizer on galanthamine content. Not only was the total amount of alkaloids markedly increased by fertilizer, but also the percentage of galanthamine in the alkaloid fraction [41].

Lubbe *et al.* [42] have also reported the effect of fertilizers on galanthamine and metabolome of *Narcissus*. They treated plants in the field with different amounts of fertilizers like 110 kg/ha of nitrogen fertilizer and 150 kg/ha of potassium fertilizer. These amounts are also applied in normal cultivation conditions by the bulb growers in The Netherlands. They also applied excessive amounts of nitrogen fertilizers (normal + 110 kg/ha) and potassium fertilizer (normal + 150 kg/ha) as a treatment to check the effect of excessive fertilizer. From the results, it was found out that the normal amount of fertilizers which is given to plants in The Netherlands is best for maximum galanthamine production (3.6 mg/g DW). They also confirmed that excessive amounts of nitrogen fertilizers are not favorable for the bulbs as it leads to soft bulbs, which are prone to diseases. Moreover, excessive amounts are not recommended due to harmful effects on the environment.

Bock with co-workers [43] studied the effect of climate variables on the first flowering on 26 varieties of Narcissus overa period of 25 years. According to their findings, temperature play an important role in the early flowering of early varieties while late varieties did not show much variation.

Recently scientist [44] conducted a survey in England to fine out the relationship between soil temperature at the time of first fertilizer application and its effect on the production and flowering of *Narcissus* plants. According to this survey's results, it is best to apply fertilizer to the plants after the first flower and not before that.

Effect of *In Vitro* Practices:

In general, plants can be effectively regenerated from a single cell or group of cells. This type of plant regeneration is called micropropagation and it is very effective to increase the multiplication rate of vegetatively propagated plants such as *Narcissus.* Other than higher multiplication rate with micropropagation, it is also possible to obtain disease-free plant material [45]. Micropropagation techniques are used in *Narcissus* solely because natural vegetative propagation of these plants is very slow (1.6 fold per annum). A number of micropropagation methods have been developed for this purpose, using the leaf, root, scale and stem explants. Callus derived from the walls of the ovary has also been used for this purpose [46-48]. Some methods give rise to rooted shoots that can be planted, while others produce bulblets. Every method used has some advantages and some disadvantages. A drawback of using underground plant parts as explant is the higher frequency of infection with soil microbes [49].

Although multiplication rate was increased, the main drawback found in the early research was that transplanting explants in the soil were not very successful. In the beginning, the cause of the poor transplanting was not known but later it was found out that leaf senescence is the major hurdle for better growth after transplanting. It was also determined that 0.20 g is the required weight of the bulblet for successful transplanting from culture to the soil [50]. Chow *et al.* [51] have reported that by trimming the green leaf tissue at alternate transfers, the multiplication rate can be increased. It is possible that there is some difference in the bulblet formation due to the explant. To check this, a comparison between the bulblets formed by single leaf explants and shoot clump cultures was made. The results showed that bulblets form slowly in the single leaf explants but respond to the growth regulators. Thus it can be useful to investigate the regulation of initiation and development of *Narcissus* bulbs [52].

Usually, bulbs formed in *in vitro* propagation only produce flowers after several years of propagation [53]. Santos *et al.* [54] managed to produce *Narcissus* bulblets flowering within the first year, after about 9 months of growth. Different types and concentrations of sugars, as well as the osmolarity of the medium, can also affect the development of the bulblets in different ways [55]. Somatic embryogenesis has also been performed with various *Narcissus* species and cultivars, using explants from various tissue types [56, 57]. Malik [58] 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 the highest number of embryos obtained directly from the explant were with an 8-week culturing system on liquid media containing growth regulators 2,4-Dichlorophenoxyacetic acid (2,4-D) and Benzylaminopurine (BA). The results of this study also show good potential for liquid media in *Narcissus* micropropagation. Micropropagation of *Narcissus* shows potential for efficient multiplication of the plant and continues to be the focus of many investigations. During the past 35 years, considerable efforts have been made to establish *in vitro* production of galanthamine by means of plant cell cultures.

Bergonon *et al.,* [59] have reported, that the production of galanthamine in shoot clump cultures was inhibited by the addition of *t*-cinnamic acid (a precursor in the biosynthesis of the AA), but it promoted the production of another alkaloid in the same biosynthetic pathway, *N*-formyl-norgalanthamine. They used *Narcissus confusus* as source plant material. They have found, that galanthamine was strongly released into the liquid medium from where it can be easily recovered. They also reported on the release of galanthamine to be higher during the light and lower during the dark period of time, opposite to the other alkaloids (haemanthamine, *N*-formylnorgalanthamine and tazettine). The total production of galanthamine in the control cultures in day-long (16 h light-8 h dark) photoperiod was 2.50 mg per culture, of which 1.97 mg per culture were released into the liquid growth medium of shoot clump cultures [59].

In another experiment shoot clump cultures were established from the bulbs and mature seeds of *Narcissus confusus* Pugsley in a liquid medium. Then these cultures were treated with different concentrations of sucrose. It became clear from the results that alkaloid production was growth associated in the shoot clumps derived from the seed (9% sucrose treatment showed good growth and alkaloid accumulation) while in the bulb-derived shoot clumps production of alkaloid was not associated with the growth. This study also showed that galanthamine production was significant at two sucrose concentrations (6%

and 18%) in bulb-derived shoot clumps [60]. There could be other factors than sucrose, which affect the production of galanthamine in the *in-vitro* cultures. Selles *et al*. [56] attempted to find the correlation between differentiation state and alkaloid content including galanthamine. They reported that dedifferentiated callus was not very good for the alkaloid production, but they can be a good system for studying the alkaloid biosynthesis. They also declared that cellular organization can influence the alkaloid profile *i.e.* embryogenic callus accumulated galanthamine type alkaloids while meristematic callus accumulated mainly haemanthamine type. They used *Narcissus confusus* as a source plant material and claimed that the embryogenic callus may accumulate the same amount of alkaloids as shoot clumps. Shahin et al. (2018) reported that with increasing differentiation of calli the galanthamine levels increases, further confirming the fact that undifferentiated plant cell cultures are not promising for improving galanthamine production.

Colque *et al.* [61] used biotic elicitors like methyl jasmonate, arachidonic acid, chitosan and salicylic acid to treat shoot clump cultures of *Narcissus confusus*. They found, that high doses of these compounds affected the growth of the explants negatively, particularly the salicylic acid. They also found that methyl jasmonate promoted the galanthamine production at all tested concentrations. Results from this study showed that the lowest amount of methyl jasmonate (25 μM) caused the release of a maximum amount of galanthamine, which was 3.8 fold more than from the control at the 10^{th} day of culture.

Diop *et al.* [62] reported that *in vitro* organogenesis can enhance the accumulation of galanthamine in the bulblets. All these experiments have been carried out at small scale. Production was too low for success at commercial scale production. Even the production capacity of cell lines often declined over time or during scale-up. Berkov et al. [24] gave an overview of all galanthamine producing plants that have been studied for in-vitro production of galanthamine. Considering the poor results the need for exploring biodiversity for other sources of galanthamine was stressed. In a later review an update was given on the research on the production of galanthamine in *in-vitro* systems [63]. The major conclusion was that the level of production was far too low for an economical biotechnological process. Best levels of galanthamine (1.7 mg/L of *Leucojum aestivum* shoots in bubble column) were obtained in differentiated cultures, which are difficult to scale up. Most importantly, they have reviewed almost all processes till date of in vitro Gal biosynthesis including growth regulators, medium components, culture conditions, elicitation, and bioreactor systems. This review can be used as a starting point for further studies in this area leading to a progress in biotechnological production of this valuable alkaloid [63].

Plant-plant interaction

Narcissus plant's interaction with other plants can be discussed in different ways. It can be divided into three categories which are the interaction between *Narcissus* plants itself, with the weeds and with the preceding crops. All these interactions have some kind of effect on the production of Narcissus crop in one way or the other but their effect on alkaloid production is not fully known yet. Almost all of the plants have to compete for their space in the field with other plants. So Narcissus is not an exception in this regard. Like all the other bulbous plants, *Narcissus* bulbs contain quite some amounts of storage compounds which are used for the early stage development of the plant from the bulb. In *Narcissus*, the initial stalk is already formed within the bulbs at the time of planting, thus there is very little effect of adverse conditions in the first season of growth. Opposite to this fact, plant density in the field can have adverse effects on the crop even in the first season.

The total plant density in the field can be divided into different forms such as the amount of the bulbs planted in the field as seed and number of weed plants present in the field. The plant density is also dependent on the vigor of the bulbs. In the UK, there is about 12.5-17.5 t/ha increase in the bulb weight after two years of bulb planting. It can be different for different cultivars [64]. Studies have shown that when planting density was higher, there was an increase of 20% in the stem length in the first year after planting. Higher density also affects the foliage of the plants which senescence more quickly in these conditions. Rees et al., [65] stated that flower numbers are only affected by bulb size when crop density was higher. It was also clear from their study that higher plant density has very little effect on the flower size in general. There is not much data available about the effect of higher plant density on flower initiation in the plants. To further explore this, Moraes-Cerdiera et al., [66] evaluated four cultivars of Narcissus with different planting depths in the field and planting density. It is evident from their results that both factors have some effect on total bulb yield. They concluded that the bulbs which were planted at 20 cm depth had better growth as compared to the bulbs that were planted at shallower depths. They also found that plant growth and yield of all four cultivars was reduced under higher plant density conditions. In terms of galanthamine contents, it was stated that there was no significant difference in these four cultivars as a response to higher plant density or planting depth. Although, there was some variation in galanthamine contents due to total biomass available. Except for this single study, there is no information in the current literature concerning the effect of planting density on the *Narcissus* metabolome and alkaloid production.

Number of weed plants present in the field is another factor which can considerably affect the plant density in the field and increase the competition for available space and nutrients. There are a few studies in which a comparison was made between the total yield of unweeded and hand-weeded plots. The results clearly show that yield of the unweeded plots was 4 to 17% less than the hand weeded plots [67-71]. Lawson and Wiseman [72] have reported in another study that the total bulb yield in the unweeded plots was 13-24% less as compared to the fully weeded plots. They also reported that the bulb yield was reduced from 7-14% in the plots which were weeded early in the season in comparison with the normal weeded plots. It was also stated in the report that the yield was reduced to 34% when there was severe stress of moisture in the spring season. All available data is about the crops which were lifted from the field on yearly basis. There is not much data published on the effect of weed competition for bulbs left in the field for one more year. Lawson and Wiseman [73] have reported in a second study that there was a loss of 4.2 to 6.4 tons per hectare when the plots were totally unweeded and 1.7 to 3.7 tons per hectare loss was recorded in the plots which were weeded only early in the season as compared the totally weeded plots. They claimed that the most serious loss was in the bulbs with large circumference (>16 cm). These bulbs were reduced by 38-63% showing that weeds are affecting the most valuable part of the plant. They have also reported that there is not much effect of the weeds at the flowering date. Other than affecting the bulb weight, weeds can also cause early senescence in the *Narcissus* plants. It can be avoided by weed removal in May and early June, as this is the time when most of the bulbs gain weight [74].

Season of the weed growth is an important factor to control the effect of weeds on the crop. The effect of weeds on the *Narcissus* plants in early spring is quite negligible. Moreover, there was almost no effect of weeds during the first year of flowering. Although severe shading by weeds in May and early June has a positive effect on the length of stem and leaves of plants, it has a negative effect on the total bulb growth [75]. In another study it was stated that May and June are the most important months in the whole growing season of *Narcissus*. This is the time when flowering ends and senescence started. Presence of weeds in these two months can quicken the senescence of the plants. During this time period it was also determined how severe the competition between crop and weeds is on the basis of duration and density of weed cover. Normally in *Narcissus*, the effect (quick senescence, fewer flowers, low yield) was amplified when weeds were very dense and in the early period of crop growth [73].

There are different seasonal weeds which have various effects on *Narcissus* plants during their growth cycle. On the basis of season, overwintered weeds show a great effect on *Narcissus* crop due to their parallel growth cycle in spring and early summer. On the other hand, weeds which germinate in the spring effect only the latter part of the crop during their growth cycle. Although the spring germinating weeds come too late in the season to affect the crop extensively, still much of their effect on the crop have not been explored yet.

Lawson and Wiseman [73] reported that variations at the start of the competetion, as well as the intensity of the competition for nutrients and available space, are caused due to the amount of overwintered and spring-germinating weed species in the field. According to their report, the total weight of weeds was always higher in the presence of overwintering weeds and lower otherwise. It was also stated that if the overwintering weeds are large, a thick weed cover is formed which shades the crop early in the season. They concluded from the above-mentioned study that it is important to eliminate overwintering weeds in the *Narcissus* crop to avoid loss in bulb yield due to plant density and shading. The elimination can be achieved either by agronomic practices (hand weeding, shallow plowing) or by the application of chemicals (herbicides) prior to the crop emergence.

Besides of the season of germination, there are specific weed species which can have some detrimental effect on the *Narcissus* crop in the field. In terms of specific weeds species, *Stellaria media* (chickweed) is the most important overwintering annual species. This weed is native to Europe. It mostly affects the *Narcissus* and spring cabbage crops due to its ability to grow rapidly from March onwards. Moreover, it has a very invasive nature due to its climbing and smothering habit that can quickly damage a whole field [76]. *Poa annua* and *Capsella bursa-pastoris* are other species of weeds which emerge in quite considerable numbers during the autumn season. The limiting factor for these species is that they are themselves subjected to competition from each other as well as other weed species in the early summer season. Due to this factor and limited time to cause damage, they seem to have no significant effect on the crop. Of the overwintering weed species particularly *Agropyron repens* competes severely with the crop, but the regular removal of its foliage during the autumn and winter markedly delayed its development in spring.

Weeds, which germinate in the spring, can also have some effect on the crop, but this effect would be little as they have a shorter period for competition with the crop. Few weed species such as *Galeopsis tetrahit, Fumaria officinalis*, and *Papaver rhoeas* can have a large effect as these species are tall and tend to germinate early in the season and grow very rapidly. But if these species germinate in autumn, there is almost no effect as they cannot survive in the winter. There is also another important fact described by Lawson and Wiseman (1972) that if Pyrazone/ Chlorbufam is applied to a mixture of weeds, all the weeds were killed but pure strands of *Galeopsis tetrahit* in one experiment and *Fumaria officinalis* in another experiment developed due to this application. They also have found that there is no evident change in the yield when compared with the unweeded plots in the experiment.

According to Rees [77], any reduction caused by higher plant density can be compensated by a higher number of plants in a unit area but the losses caused by the competition of weeds with the *Narcissus* plant cannot be recovered due to the presence of weed plants in the crop. A few studies have been done to check the causes of the decrease in bulb yield. According to these studies, the effects of weeds are not the only cause that decreases the total bulb yield. In these studies, bulbs of equal size were lifted from weeded and unweeded plants after one year and then those bulbs were forced into the greenhouse. The outcome of this showed that there are clear differences in the number of flowers produced and the quality of flowers. Thus it cannot be said that weeds are the only reason decreasing the bulb yield [70-72, 76]. Lawson and Wiseman [73] concluded that different effects of weed competition occur, depending on variations in soil and weather conditions, weed species and density, and crop growth. There will be further differences in commercial practice due to crop cultivar and size and a planting density of bulbs. It is, therefore, difficult to predict accurately the likely crop losses due to weeds in commercial production, though a probable range seems to be 5-20%.

To conclude, further work still needs to be done to understand the complex relationship between weeds and Narcissus crop. In fact, nothing is known about the effect of competition between weeds and Narcissus on the metabolome of Narcissus. It would be quite interesting to study the effect of weeds on the metabolome and galanthamine content in Narcissus.

The case of *Narcissus* interaction with other crops, which are planted before or after the crop, is also complex. Barley, leys, peas and early potatoes are cultivated mostly in a rotation with the *Narcissus*. It is important to note that high residual crops such as cauliflower must be avoided in the rotation because high nitrogen contents may boost the basal rot disease in the bulbs [78].

In a recent study researchers [79] have tried to grow *Narcissus* on marginal lands to improve production and utlilize the land at four different sites in England. According to their finding, around 80% bulbs were established at each site from the planted material. Moreover these established bulbs have higher galanthamine contents as compared to the other locations. They have also found out that altitude and planting density have almost no effect on the galanthamine contents as well as on the production of bulbs. A limited amount of information about the effect of other crops on the soil contents in relation to *Narcissus* can be found in the handbook of *Narcissus* and Daffodil [41]. But there is hardly any information available about some change in galanthamine contents or metabolic changes due to crop rotation or cover trees. It is quite possible that there are some changes in the metabolome of *Narcissus* due to these interactions with other plants in the field that are still left to be explored.

Plant-insect interaction

Narcissus is entomophilous (insect pollinated) crop and the likelihood of a flower being pollinated probably depends upon the weather and its effect on pollinating insects during the flowering season [41].

Plant beneficial Insects:

Due to their sessile style of life, flowers have limited possibility of spreading their pollen. That is the main reason for the necessity of pollen vectors for successful pollination in flowering plants. Among pollen vectors, animals represent nearly three-quarter of all pollen vectors in *Angiosperms* [80]. Although much work has been done in this field, the detailed understanding of principles of pollination via attracting insects to a particular flowering plant remains cryptic. The question is very complex and involves diverse viewpoints and specializations [81]. The role of secondary metabolites in the attraction or repellency of insects has been discussed in these reports [82, 83]. Though *Narcissus* species propagate via bulbs, the yellow scenting flowers attract a number of pollinating insects. Except *N. longispathus*, most *Narcissus* species are self-sterile [84] and are therefore likely to be dependent on pollinator services of insects and other animals. Human disturbance is one of the factors negatively impacting plant-pollinator interactions [85].

As debated by Miller *et al*. [86], the typical spring color of pollinated flowers is blue and purple. “Yellow flowers have the ability to stand out from the denser green vegetation in the late summer”. According to this hypothesis, *Narcissus* has a significant advantage among other spring flowers. The yellow color of *Narcissus* species is due to the carotenoids in the inflorescence. Carotenoids hold diverse functions in plants varying from an essential role in photosynthesis to precursors for signaling molecules. Moreover, they are a source of vitamins for humans. Besides of pro-vitamin A source, carotenoids are used as pigments, antioxidants, food/feed additives, cosmetics, pharmaceuticals, nutraceuticals, and dyes. Some flowering plants, such as red marigold flowers, are used as a commercial source for zeaxanthin extraction reviewed by Farrè *et al*. [87]. In *Narcissus*, xanthophyll has been detected by Kuhn and Winterstein [88] in *N. pseudonarcissus* and crystals of *β*-carotene were first observed in the corona of *N. poeticus* [89]. From *Narcissus* "Golden Harvest" a number of carotenoids were isolated by Berset and Pfander [90] with the major compound (all-E)-lutein. Some genes of the carotenoid pathway are isolated from *Narcissus* and subjected to further expression in diverse systems such as insects or plastids (**Table 1.1** mentioned above). The relevance of *Narcissus* carotenoids is shown in the beginning of the famous 'Golden Rice' project, for which a gene from the carotenoid biosynthesis pathway was isolated from *Narcissus pseudonarcissus* and introduced successfully into rice to introduce the vitamin A pathway [91].

It is known that insects don't perceive the colors in the same way as we do. For example, bees are able to see in ultraviolet light [92]. If one puts the petals of *Narcissus* flowers under the UV light, interesting patterns can be observed. The tip of each petal shows blue fluorescence due to the presence of a tuft of trichomes. The function of such a dartboard as a nectar guide for UV-sensitive pollinators has been the subject of discussion for many scientists as the fluorescence is rather far from the actual nectar reservoir [93]. Hence the role of additional insects routing via scent is proposed. In general, trichomes comprise unique structures which are the site of biosynthesis or storage of (secondary) metabolites [94]. Lam with coworkers [95] studied the morphology of *Narcissus tazetta* trichomes and also performed a couple of chemical reactions in order to uncover the nature of compounds present in the trichomes. The presence of flavonoids and/or phenylpropanoids was revealed. Some "apical hairs" were reported also in *N. incomparabilis* and *N. pseudonarcissus* [96]. From flowers of *N. tazetta*, two compounds namely isorhamnetin (flavonoid) and tazettine (alkaloid) were isolated by Shikhiev and Serkerov [97].

Regarding the colors as attractants for the pollinators, beside petals, other parts such as stigma, pollen and nectar could be colored or in contrast against the background, thus be visible for insects as reviewed in Miller et al. [86]. However, many present-day *Narcissus* color variations are the result of intensive breeding and thus they did not undergo pollinators' selection. This is because daffodils propagate via bulbs so seed production is not desired. On the contrary, *Narcissus* for multiplication purposes are treated (physically and/or chemically) not to spend energy on seed production. New variety development is done via man-crossing and doesn't depend on precarious pollinators services. But, the non-pathological natural interaction between daffodils and insects is described here, though the number of reports is found to be scarce.

Other insect appealing metabolites produced and/or occur in flowers are compounds of volatile character. Their role is mostly attributed to attracting pollinators, however, other roles such as in defense [98] and way of communication between the plants [99] has been proposed. Sometimes flowers employ a rather sophisticated strategy in attracting pollinators via scent. For example, some orchid flowers produce volatiles similar to honey bee [100] or green leaves volatiles signaling leave damage (by e.g. herbivorous insects) [101] in order to attract those insects predators [102]. According to Pérez-Barrales *et al.* [103], the insects pollinating *Narcissus* are strongly influenced by the anatomy and herkogamy of individual *Narcissus* species. The role of chemical constituents of the *Narcissus* fragrance in the attraction of pollinators has been studied by Dobson and coworkers [104]. No unique pollinator such as in the Orchids case has been appointed through some sort of grouping of daffodils into Lepidoptera and non-Lepidoptera odors has been observed. This phenomenon was in agreement with previous observations made by Knuth at the end of 19th century in Dobson *et al.* [104]. Insects of non-Lepidoptera odors are mainly bees (Hymenoptera) and some flies (Diptera) and their fragrance is more heterogeneous. Whereas the Lepidoptera group represents mostly moths and butterflies and their "Lepidoptera odor" principle is observed in diverse flowers pollinated with mentioned insects [105]. The Lepidoptera type of odor was characterized in *Narcissus* by the presence of indole, a high percentage of esters (both terpenoid and benzenoid), linalool and *trans*-β-ocimene. Plants producing scent poorer in this "Lepidoptera mixture" and richer in diverse benzenoid esters that belong to an intermediate group in which an extension of no detectable indole creates the non-Lepidoptera odor type [104]. *Narcissus* scent production is one of the breeder's concerns. Decorative flowers scent is both desirable and unwelcome, depending on the intensity, scent characteristic and also customer's requirements or local habits. However, the visual appearance and shelf life of flowers is of higher priority than the scent. In general, selection of longer shelf life flowers resulted in less fragrance producing flowers [106]. Besides of the decorative aspect of daffodils, the scent of *Narcissus* is of interest for extraction. *Narcissus absolute* is commercially obtained from *N. tazetta*, *N. jonquilla* and mainly *N. poeticus*. This essential oil is considered rare and expensive. About 500 kg of flowers is needed to produce 1 kilogram of concrete or 300 g of absolute oil. In spite of the scarcity, about 70% of women's perfumes sold nowadays contain a note of *Narcissus* absolute.

Harmful Insects:

Harmful insect includes only the large *Narcissus* fly (*Merodon equestris*). In the UK bulb industry, the large *Narcissus* fly is referred as the major insect pest. To improve the yield and quality as well as to produce blooms with long stocks, bulbs in the UK are left in the field for 2-3 years. This is not the case in The Netherlands where bulbs are lifted from the field after one year. But still, the large *Narcissus* fly also causes damage in The Netherlands. There are three species of this pest. First (*Merodon equestris*) is large while the other two species (*Eumerus strigatus* and *E. tuberculatus*) are quite small. Most of the damage is caused by the large fly, which can attack healthy bulbs, while the smaller ones can only attack damaged bulbs.

This pest causes damage by laying a single egg on the neck of the bulb. Favorable conditions for this pest are bright, still sunny days in which temperatures are above 18 °C. Most of the egg laying occurs under these conditions. A single fly can lay eggs at the necks of up to 100 bulbs. The egg hatches after the egg-laying period which is from the beginning of May until June. When eggs hatch the larvae crawl to the base of the bulbs. From the base this pest tunnels inside of the bulb and feed on the fleshy leaves [107, 108].There is a previous report about the effect of temperature on this pest by Which [109]. According to this report, low temperatures during the above-mentioned time period affect the egg laying ability of these pests.

Narcissus is also affected by Nematodes present in the soil [110]. Mostly the stem and bulb parts of the plant are affected during their growth period. The main causal agent in *Narcissus* is *Ditylenchus dipsaci*. In this disease, the leaves of the plant are stunted and distorted. Leaf center and margins are swelled and become yellow or brown colored. Sometimes leaves are thickened and swollen at the base. The irrigation water spreads nematodes. They also survive in weeds and even can survive desiccation. Low temperatures are quite favorable for nematodes. For controlling this disease, the planting equipment needs to be cleaned. Moreover, infected bulbs need to be removed from the field to control the spreading.

Other animals:

Wild rabbits and other rodents such as rats are usually somewhat harmful to the crops. Rabbits eat mostly the leaves of the plants and rats damage the roots and bulbs of the crops. The observation made in 40 farms in the UK revealed that rabbits cost the farmers about 0.65 million British pounds damage per year (Defra, 2003). However, *Narcissus* leaves and scapes are damaged only to a very small extent. In one area, *Narcissus* was grown near a hedge bank which was infested by rabbits. No *Narcissus* damage was noted in that field as they run past to the part of the field where there were no *Narcissus* plants. In another area, rabbits caused extensive damage by nibbling on the leaves of *Endymion nonscriptus* but the leaves of *Narcissus* and *Orchis mascula* growing in the same area were left untouched. On the other hand, slugs can eat flowers of *Narcissus.* Coldwell and Wallace [111] examined about 82 flowers in total in a badly drained pasture after wet weather. From those 82 flowers 56 were mutilated. They have found that slugs and snails cause a small amount of damage occasionally.

Grazing animals such as cows, goats and sheep affect unprotected crops and wild plants by eating their leaves and stems, thus causing a great deal of damage. There are reports that these grazing animals do not damage *Narcissus* plants. The wild *Narcissus* and even cultivated varieties are not eaten by cows and goats. Clearly, they even avoid the leaves if they are in the middle of a field. Chicken peck a little on the flower buds but the leaves are left totally unaffected. While pigs grouted in only those areas where there are no bulbs in a patch of 2 acres which was covered with *Narcissus* [111].

It can only be speculated about the reasons why animals and most insects do not like to eat *Narcissus* in the field. There can be different reasons for this such as the structure of the *Narcissus* leaves and plant, the presence of trichomes and last but not the least the presence of anti-feeding compounds.

Concerning the structure of the *Narcissus* plants and the leaf surface, it is possible that the presence of wax is the cause that insects avoid the leaves of this plant. The cuticles of plants are covered with hydrophobic constituents. These coatings are the first layer of defense of plants against herbivores. Eigenbrode and Espelie [112] postulated that the glossy surface of the leaves causes insect resistance in most of the *Brassica spp*. especially in *B. napus* and some other crops.

The presence of trichomes can be another factor in the defense of *Narcissus* plants against insects and animals. They can be unicellular and multicellular and arise from epidermal cells of plants. They are present in *Narcissus,* so they could play an important role in plant defense against small insects. Trichomes provide an effective barrier to the movement and contact of insects and also deter them from egg laying [113]. Thus the presence of a large number of trichomes can provide some defense against sucking or piercing type of insects [114]. Norris and Kogan [115] have described the role of trichomes in protecting different crops from a number of insect *spp*. In *Narcissus,* Lam and coworkers [95] studied the morphology of trichomes. They have also performed some chemical reactions to find out the nature of the compounds that are present in the trichomes. According to their findings, flavonoids and phenylpropanoids are main compounds which can be found in the trichome of *N. tazetta*.

Volatiles can be an important factor for the defense of *Narcissus* against the animals and the insects. They may repel the animals from the *Narcissus* plants. Different kinds of volatiles are emitted from the floral and vegetative parts of plants. Thus, it can be that *Narcissus* emits some compounds from their flowers or maybe from their vegetative parts which act as a warning for the animals and insects, so they stay away from the *Narcissus* plants. There are reports that plants protect themselves from herbivores by emitting high levels of *S-methyl-methionine.* Isoprene is a simple C_5 terpenoid volatile that is released from non-floral parts of plants [116]. There are also reports about a defensive role of polyphenol oxidases against herbivores. De Moraes and coworkers [117] stated, that after the damage of green leaves of tobacco, volatile (*Z*)-3-hexenyl acetate and aliphatic esters of (*Z*)-3-hexen-1-ol are emitted which repel the female moths from egg laying on these injured plants. There is a study in which it is described that volatiles from *N. attentuate* act as a direct defense by repelling the herbivores and indirectly by attracting the predators of these herbivores. Vancanneyt and coworkers [118] found that volatiles from the green leaves of potatoes may be involved in the resistance against aphids. So, it is possible that volatiles of the plants contributes to defense against the animals and insects.

Volatiles from plants can influence other plants in their vicinity as well. It seems reasonable that plants can perceive the volatiles produced after the damage by herbivores and respond to this information by activating their own defenses. It can be the case that *Narcissus* plants receive signals from other plants in the nearby grazing area and they produce and emit chemicals which can repel animals. For example, when *Vicia faba* plants were exposed to (*Z*)-jasmone, they emitted the volatile metabolite methyl jasmonate which caused elevated levels of the monoterpene *β*-ocimene in neighboring *V. faba* plants [119].

However, there is little information available on volatiles in *Narcissus* and their effect on animals in the surrounding area. Headspace analysis may provide information about possible defense mechanisms of *Narcissus* plants. Previous studies using headspace analysis collected the volatiles from the flowers of *Narcissus poeticus* and *N. tazetta* [120, 121] but no attempt is reported about the analysis of volatiles from the vegetative parts of the *Narcissus* neither is there any study of possible repellent effects.

Diseases of *Narcissus*

Diseases are an important factor to consider improving the yield of plants in the field. All crops are affected by diseases in one way or another during their growth cycle. Some crops can be severely affected while in the others damage can be limited. *Narcissus* is also not a disease-free crop and at times it is affected rather severely. Sometimes more than 50% of the bulbs are affected during storage and even on occasion if the diseases are not controlled properly they can damage a whole field within a short time. Hence it is important to know which diseases can affect the crop and how much. Some of the major diseases of *Narcissus* are discussed here.

Fungal Diseases:

Top of the list in fungal diseases of *Narcissus* is rot which has different types. Basal rot is a fungal disease which is caused by a specific organism for *Narcissus.* The causal agent for this fungal disease is *Fusarium oxysporum f. sp. Narcissi*. It is a type of rot which starts from the basal plate of the bulb (hence the name basal rot) and moves upwards to the stem and leaves. This disease affects the crop severely in moist and warm weather while the effect in cold conditions is quite limited. The fungus can survive in the form of chlamydospores. High nitrogen concentrations in the soil or nitrogen fertilizers are quite favorable for this fungus. Symptoms of basal rot include yellow and distorted leaves and stunted growth of the plants, which usually lead to the death of plants. The infested bulbs have reddish brown colored basal plates, which are decayed. The bulbs rot in the field and even in the stores by this pathogen. Sometimes white to pinkish moulds can be found between the layers of the bulbs. Carlton and Golden Harvest varieties are quite susceptible to this disease while St. Keverne is resistant to the disease. This disease is rather difficult to control in the field. For controlling the disease, bulbs are lifted as early as possible after the infection and stored below 13 °C. This disease can also be controlled by Hot Water Treatment [122-124]. At the places where a straw cover is used for the protection of crop from frost, leaving the cover in place may reduce the amount of basal rot by decreasing the temperature of the soil [125].

Crown/neck Rot is almost like the basal rot except that it starts from the crown/neck part of the bulb and moves downward to the basal plate. This disease is caused by *Sclerotium rolfsii*. At the start of this disease, the bulbs become soft and damp which almost looks like waterlogged. After some time and with the progress of this disease, they become dry and turn into ‘woody’ bulbs. The mold formed by this fungus covers the whole bulbs in coarse white layers. Warm weather is quite favorable to this pathogen. The fungus survives in the soil for long periods of time and sometimes even up to 10 years. This disease can be controlled by avoiding the infested fields for 4-5 years. It can also be controlled by deep plowing of the soil and Hot Water Treatment (HWT) of the bulbs before planting in the field [126].

White root rot is also a fungal disease of *Narcissus* which is caused by *Rosellinia necatrix*. This fungus has a wide host range and can invade the plants by the roots or from the stem bases. This disease can cause bare patches in the field i.e. the bulbs cannot produce the shoots. Periods of mild wet weather are favorable conditions for the spread of this disease. This disease can only be controlled chemically [127].

Black rot is caused by *Sclerotinia sclerotiorum*. In this disease, thin gray masses of the mold cover the bulbs with black irregular sclerotia between scales. Cool temperatures are favorable to this fungus and infection can be very low in hot weather. For the control of this disease, infected plants need to be removed as soon as possible with the soil surrounding the bulbs. Clean and healthy bulbs need to be planted in the clean soil. The pathogen would be dead without a suitable host after 2 -3 years [128].

Blue mold is a disease which occurs during the storage of the bulbs. It is caused by *Penicillium corymbiferum spp.* This disease occurs mostly in the damaged bulbs. It can be avoided by removing the bulbs which are damaged [129-133].

Fire is a type of fungal disease in which the attack comes from the tip of the flower to downwards. The causal agent for this disease is *Botryotinia polyblastis*. In this disease, the areas of the petals which are soaked in water are affected first and then the foliage of the plant is affected. The pathogen of the disease survives in the debris of the leaves. This disease can be controlled by removal of flowers before it can produce apothecia. A proper fungicide spray can also prevent the disease [134].

In Great Britain, another disease which is sometimes also named as fire is more commonly known as scorch. Its causal agent (*Stagonospora curtisii*) is also different from the fire disease. The tips of the early leaves appeared to be scorched as early symptoms of this disease. The early symptoms are sometimes misunderstood as frost burns. Dampness helps in spreading of this disease while hot and dry weather tends to stop it. Grand Soleil d'Or, Medusa and Cheerfulness are the most susceptible varieties to this disease.

Smolder is a disease caused by the *Botrytis narcissicola* fungus. This disease has early symptoms like a dark brown lesion appearing at the tip of the leaves or the leaves can be curled when they are infected at the inner edges. Cold and wet weather are quite favorable for this disease. It can be controlled by the early digging of the bulbs and crop rotation.

There is a need for spraying when the disease appears in case of severe infections [135-137].

Lubbe et al. (2012) studied the effect of some fungicides on the metabolite profiles of bulbs of the Carlton variety of *N. pseudonarcissus*. Bulbs from plants subjected to a number of different treatments were compared with non-treated plants for their metabolome. The different treatments all showed a different metabolome from the control. In some cases a lower level of galanthamine was observed, in others a similar level as the control. The overall conclusion was that the current practice of using fungicides in the cultivation of *N. pseudonarcissus* is suitable for the galanthamine production. Though the overall metabolome is clearly different from the wild type. It seems that each fungicide leaves a specific footprint in the bulbs.

Other Diseases:

There are also some viral diseases which affect *Narcissus.* White streak is one of them. It is caused by *Narcissus white streak* virus. It has symptoms like narrow streaks of dark green to purple which become white to yellowish white later on. These streaks appear on the leaves and at the flower stalk after flowering stage. In this disease, the plant starts senescence prematurely, so the bulbs size and yields are reduced. This disease is spread mainly through aphids and *Narcissus* is the only host for this virus. Symptoms of this disease mostly appear when the temperature is higher than 18 ˚C. To control the disease, aphids need to be controlled and infected plants need to be removed [138]

The yellow stripe is also a viral disease which is caused by *Narcissus yellow stripe* virus. Light green to yellow streaks appears in the plants as well as leaves and flower stalks are mottled. Leaves are distorted, and color breaks occur in flowers. This disease also reduces the bulb yield. The symptoms of this disease appear in the early growing season. This virus is also mainly spread by the aphids. To control the disease, infected plants need to be removed [139]. There is very little or no information available about the effect of diseases on the metabolome of *Narcissus* in general and on galanthamine in specific. Diseases may cause a change in the metabolome of *Narcissus* by affecting the production of different compounds in the bulbs or in the whole plant, but no reports have been made public on this issue.

Conclusion:

There is a lot of practical information available on *Narcissus* as a crop for flower and bulb production purposes. However, only a limited amount of information is obtainable on the secondary metabolites present in this plant and their possible role in plant resistance to pest and diseases. This also includes the major target (galanthamine) of this study. A limited amount of knowledge about the biosynthetic pathway of galanthamine and other alkaloids is available, but which does not clarify their role in the growth and defense mechanism of these plants. This all adds up to only a limited amount of data on the possibility to improve galanthamine and other alkaloids production in the field. Though there are some indications that the alkaloid level in the plant is variable during its growth cycle and this might be modified by certain measures in the cultivation as well as pre and post-harvest processing. A lot of work needs to be done to know more about the factors that initiates and control galanthamine production in the *Narcissus* plants. It can be helpful to identify the changes that occur during or after the interaction with other plants and animals in the field. Though there is some information available on the effect of insects and diseases on total yield of *Narcissus* in the field but there is no information on the metabolic effect of these insects and diseases.

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