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Chapter 9. Tulip gum as novel source of 6-Tuliposide B

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Abstract

Certain tulip cultivars produce large amounts of gum in the bulbs when infected with the fungus, *Fusarium oxysporium*. Gummosis can also be induced by applying ethylene gas or ethylene-releasing substances to the bulbs after harvest. Previously, the composition of tulip gum has been studied in terms of large macromolecules. Many studies on the composition of tulip gum reported it to consist mainly of polysaccharides. The gum polysaccharides have been analyzed to determine sugar composition and molecular mass. Up to now relatively little is known about the gum in terms of low molecular weight metabolite content. In the first part of this chapter extracts of tulip bulb gum were analyzed by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy and were found to contain 6-tuliposide B. This was the first time tuliposides were observed in the gum from tulip bulbs. Isolated tulipalins and tuliposides possess various bioactivities, such as antibacterial, antifungal and insecticidal properties. The presence of these bioactive molecules in tulip gum may suggest a protective role for this physiological response. The fact that this compound occurs in the gum in relatively pure form, means that tulip gum may be a good natural source of 6-tuliposide B and related compounds for industrial uses. In the second part of the chapter experiments are described on tulip bulbs of different cultivars to determine how various factors (ethylene and methyl jasmonate application, temperature, mechanical wounding and time) affect the gum production and tuliposide concentration in the gum. For quantitative analysis of 6-tuliposide B in the gum a quantitative ^{1}H NMR method was developed. The results show it is possible to induce tulip bulbs to produce large amounts of gum, containing 6-tuliposide B. With careful optimization of induction conditions, this method has potential for use as a production method for 6-tuliposide B as an industrial product.

Tuliposides in Tulip Gum

Introduction

The plant pathogenic fungus, *Fusarium oxysporum* causes major problems in the cultivation of tulips. Infection with this pathogen can cause severe losses, especially during storage. Infected bulbs release large amounts of ethylene gas, which can have negative effects on otherwise healthy bulbs. Such effects include flower abortion, poor rooting and excessive splitting (Kamerbeek and De Munk, 1976). Another unwanted effect is gummosis, the production of a gum-like substance that is released into the spaces between bulb scales. When a large amount of the gum is produced it can form blisters under the surface of the bulbs or be extruded to the outside (Figure 1). Gummosis has also been reported in stems of tulips (Saniewski et al., 1998).

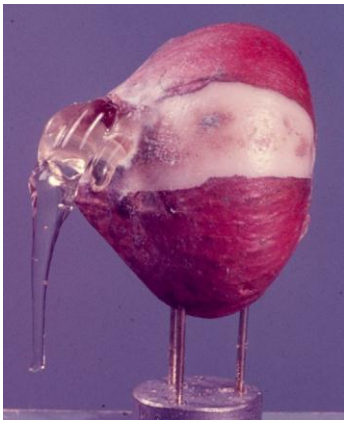

Figure 1. Gummosis in tulip bulb infected with *Fusarium oxysporum* (photo: PPO, Lisse).

Gummosis in tulips has been studied to better understand factors that induce the process, and the underlying carbohydrate metabolism involved (Saniewski et al., 2007). The composition of tulip gum has been mostly studied in terms of large macromolecules. Polysaccharides from tulip gum induced on tulip stems have been analyzed to determine sugar composition (De Munk and Saniewski, 1989) and molecular mass (Skrzypek et al., 2005). These studies showed that tulip (stem) gums consist of glucuronoarabinoxylan with an average molecular weight of ca. 700 kDa. Up to now relatively little is known about the gum in terms of small (low molecular weight) metabolite content. In this study tulip gum was analyzed by ^{1}H NMR spectroscopy to

investigate its low molecular weight metabolite profile. The aim was to determine whether the gum contained any interesting or novel small metabolites.

Materials and methods

Tulip gum (50 mg) from cultivar Apeldoorn was extracted with 1.5 mL MeOD and KH_2PO_4 buffer (pH 6), 1:1 (Kim et al., 2010) by vortexing for 30 s and sonication for 30 minutes. The sample was centrifuged and 800 µL of the solvent was collected for ^1H NMR analysis. Additional tulip gum samples were collected from bulbs of cultivar Madame Lefeber, Apeldoorn and Yokohama. These samples were extracted in the same way as described and analyzed by ^1H NMR. Tulip bulbs of two different cultivars (Santander and Flair) were ground to a fine powder in liquid nitrogen. The ground bulb material was freeze-dried and once dry, 50 mg was weighed into 2 mL eppendorf tubes. The bulb material was extracted in the same way as the tulip gum described above (KH_2PO_4 buffer in D2O -MeOD 1:1)

Results

To investigate the content of small metabolites in tulip gum, a gum sample collected from a bulb of cultivar Apeldoorn was extracted with a standard extraction solvent used in NMR-based plant metabolite analysis (methanol/phosphate buffer, 1:1)(Kim et al., 2010). Inspection of the NMR spectra revealed some high intensity signals in an otherwise relatively clean spectrum (Figure 2). Characteristic doublet signals at δ 5.19 and δ 4.59 indicated the presence of the anomeric protons of alpha and beta glucose moieties. Other interesting features were singlets at δ 5.98 and δ 5.75. Further 2D NMR experiments allowed assignment of signals and identification of this compound as 6-tuliposide B (Figure 3 and 4). Signal assignments are shown in Table 1, and corresponds well to those reported in the literature (Shoji Kazuaki et al., 2005),(Christensen and Kristiansen, 1999) , with small differences in chemical shift values due to the use of a different NMR solvent. Integrals from the anomeric proton (H-1: δ 5.19 and δ 4.59) showed that the equilibrium between the α- and β-forms of 6-tuliposide B was approximately 4:6 in this solvent.

Tulip bulbs of two different cultivars were extracted in the same way as the tulip gum (the KH_2PO_4 buffer-MeOD, 1:1) to compare the compositions of the extracts. Bulbs of cultivar Santander and Flair were compared with tulip gum from cultivar Madame Lefeber. As with tulip gum from cultivar Apeldoorn, the extract of the Madame Lefeber gum was found to contain 6-tuliposide B in a relatively pure state. The 6-tuliposide B signals were also seen in the ^1H NMR spectra of the bulb extracts (Figure 5b and c), but at about 25% of the intensity as compared to the gum signals. The bulb extracts were

more complex and contained signals characteristic of sugars, amino acids and fatty acids, and some phenolic compounds (Choi et al., 2006; Abdel-Farid et al., 2007; Kim and Verpoorte, 2010). The bulb of cultivar Santander had some prominent signals at δ 5.79 and δ 6.30, in the same region as the tuliposide B signals. A prominent triplet at δ 2.57 could also be seen with an integrated area double that of each of the other two signals. These and other signals belonging to the sugar moiety matches signals previously reported for tuliposide A in the literature (Christensen, 1995).

Table 1. Assigned ^{1}H NMR signals of 6-tuliposide B extracted from tulip gum, in MeOD and KH_2PO_4 buffer (pH 6) 1:1, measured at 500 MHz.

H	**δ (ppm)**	**Splitting pattern**	**J (Hz)**	**integral**	**COSY**
3'a	5.98	s		1	
3'b	5.75	s		1	2.92, 2.75
4'	3.83	dd	4.35, 8.05	1	2.92, 2.76
5'a	2.92	dd	14.8, 4.35	1	2.76, 3.83, 5.75
5'b	2.76	dd	14.8, 8.05	1	2.92, 3.83, 5.75
β-glucose:					
1	4.59	d	8.2	0.6	3.20
2	3.20	dd	8.8, 8.2		4.59, 3.44
3	3.44	t	8.86		3.20
4	3.52	d	11.86		3.70
5	3.70	m			3.52, 3.88, 4.03
6a	3.88	dd	12.3, 2.0		4.03, 3.70
6b	4.03	dd	12.3, 2.0		3.88, 3.70
α-glucose:					
1	5.19	d	3.75	0.4	3.48
2	3.48	dd	3.75, 9.8		5.19,
3	3.63	t	9.8		3.24
4	3.24	t	9.8		3.63
5	3.48	m			3.73, 3.69
6a	3.73	dd	12.0, 5.0		3.69
6b	3.69	dd	12.0, 2.0		3.73

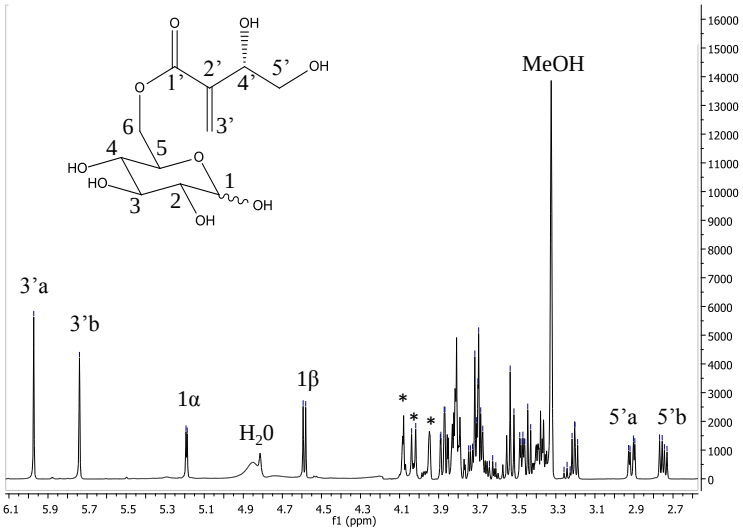

Figure 2. Structure of 6-tuliposide B and ^{1}HNMR spectrum (500 MHz) of tulip gum in MeOD and KH_2PO_4 buffer (pH 6) 1:1, with non-overlapping signals labeled. Signals not belonging to 6-tuliposide B are labeled with an asterisk (*).

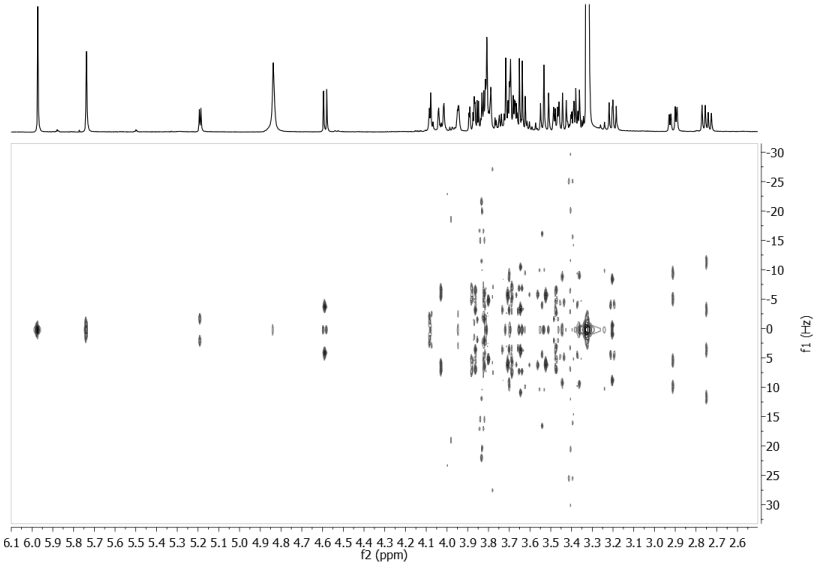

Figure 3. 2D J-Resolved spectrum of 6-tuliposide B recorded in MeOD and KH_2PO_4 buffer (pH 6) 1:1 at 500 MHz.

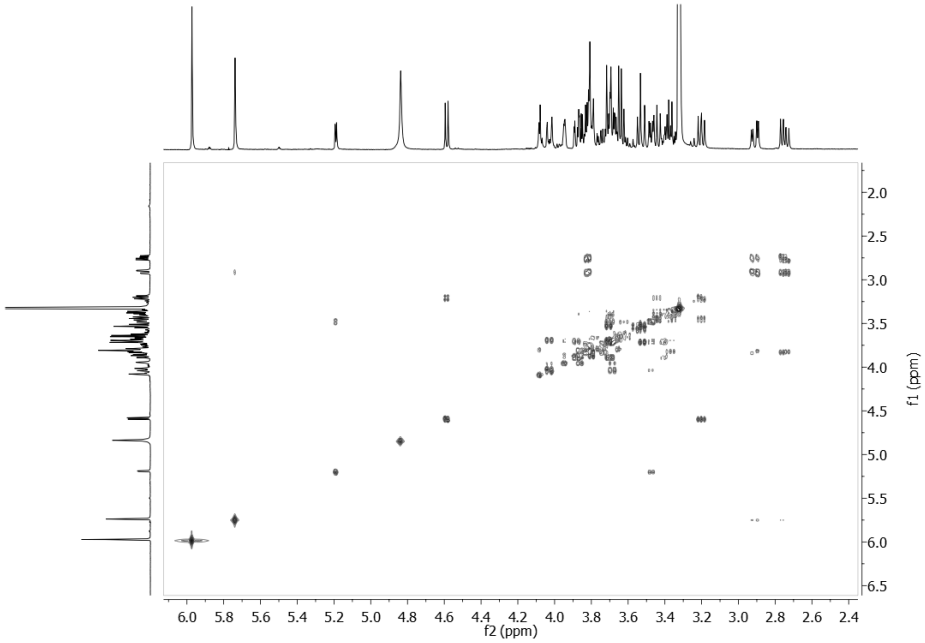

Figure 4. 2D COSY spectrum of 6-tuliposide B recorded in MeOD and KH_2PO_4 buffer (pH 6) 1:1 at 500 MHz.

The color of the tulip gum was seen to vary from clear and yellowish to opaque and brownish. Gum samples of different colors were extracted and analyzed by ^{1}H NMR to investigate their content. Two clear gum samples collected from two different cultivars (Apeldoorn and Yokohama) were analyzed. A brownish sample collected from inside a blister on the outside of an Apeldoorn bulb was also analyzed. The 6-tuliposide B was present in all three samples, with the Yokohama gum sample containing the smallest amount (Figure 6). In the clear Apeldoorn gum 6-tuliposide B was present in a relatively pure state. In contrast the brown Apeldoorn gum from the blister also contained the compound tentatively identified as tuliposide A. Two triplet signals at δ 6.19 and δ 5.82 could be seen in this sample as well. These signals are most likely due to the presence of tulipalin A (Tschesche et al., 1968, 1969; Christensen Lars P, 1999). In the aromatic region of the spectrum there are also signals that probably belong to phenolic compounds. These compounds may be responsible for the brownish color, as these signals are absent in the clear gums.

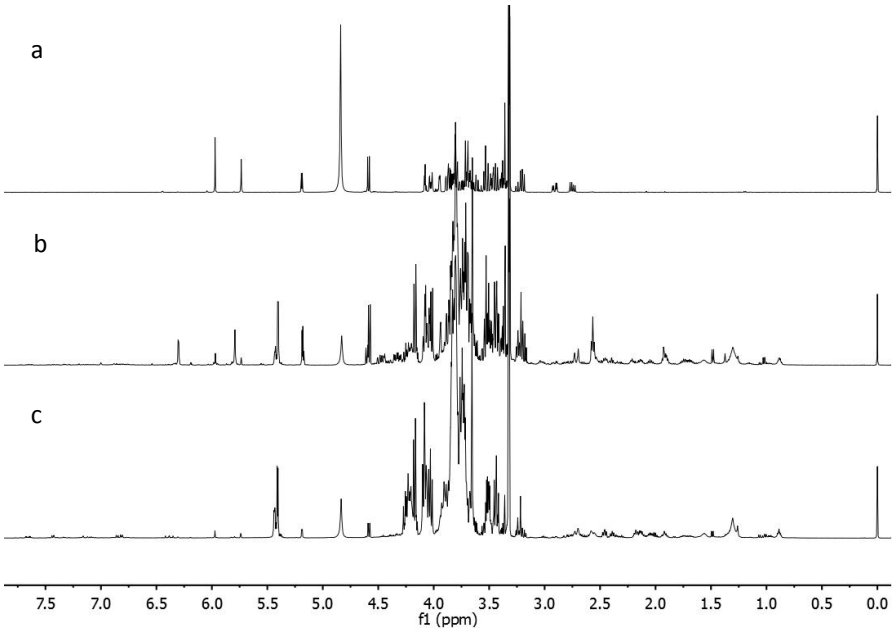

Figure 5. ^{1}H NMR spectra of extracts of tulip gum (a) and bulbs of cultivar Santander (b) and Flair (c) showing the differences in intensity of the 6-tuliposide B signals at δ 5.98 and δ 5.75. ^{1}H NMR spectra recorded in MeOD and KH_2PO_4 buffer (pH 6) 1:1 at 500 MHz.

Discussion

Tuliposides and their related α-methylene-γ-butyrolactones, tulipalins, are known to occur in various organs of the tulip plant. The presence of tuliposides or tulipalins in tulip gum has not been reported before. Studies on the composition of tulip gum in the past focused on the molecular weight of the gum polysaccharides (Skrzypek et al., 2005), and the sugar composition of the polysaccharides (De Munk and Saniewski, 1989). These analyses usually involved hydrolyzing the gum samples for several hours in acid, conditions which would likely have caused degradation of the tuliposides.

Six tuliposides and two tulipalins have been isolated from tulips. The structures of these are shown in Figure 7. Tuliposides A and B are widely distributed in the genus *Tulipa*, with different ratios of the different kinds being reported. Tulips in section Leiostemones usually contain larger amount of tuliposide B than A, while those in section Eriostemones have large amounts of both A and B with no consistent interrelationship between the two (Christensen and Kristiansen, 1999). Tuliposide D

occurs in large amounts in *T. patens*, and only in trace amounts in other species (Christensen and Kristiansen, 1999).

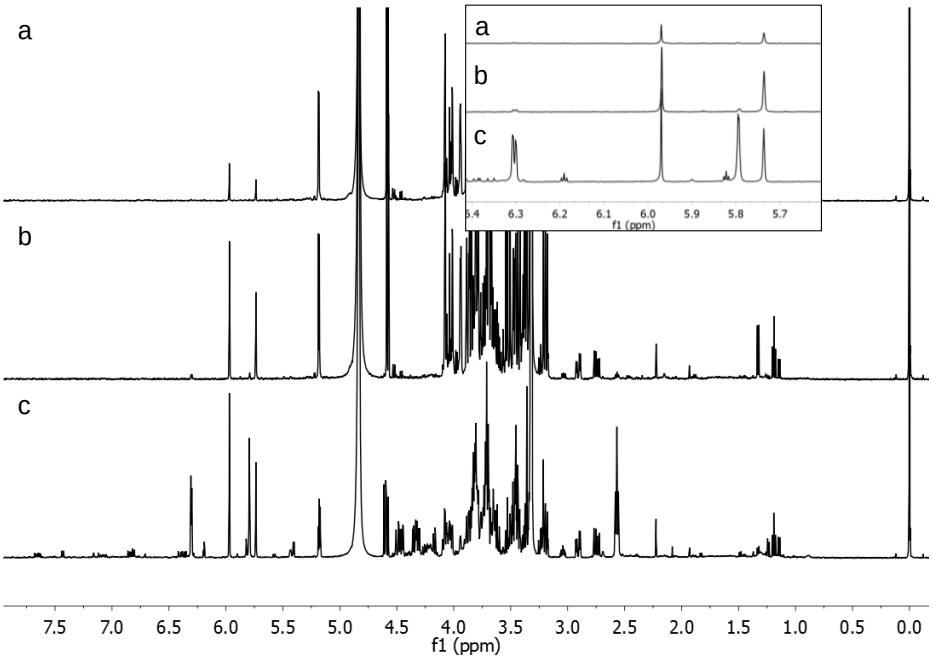

Figure 6. ^{1}H NMR spectra of extracts prepared from clear Apeldoorn gum (a), clear Yokohama gum (b) and brown Apeldoorn gum. ^{1}H NMR spectra recorded in MeOD-buffer at 500 MHz. Insert shows magnified region of spectra from about δ 6.45 to δ 5.65.

Tuliposide F was first isolated from *T. turkestania* and has also been found in all taxa investigated since. The name Tuliposide C was given to a compound from tulip in an early study, but since this compound was never fully characterized, the next tuliposide that was completely elucidated was named tuliposide D.

Tulipalins are released from tuliposides spontaneously in response to increased pH or enzymatically (Beijersbergen and Lemmers, 1972). Above pH 5.2 glucose is released from tuliposide, and the free acids lactonize to form the tulipalins (Schönbeck and Schroeder, 1972). Above pH 7.0 the free acids are reportedly present. Tulipalin A was initially isolated from tulip bulb material as an antifungal compound (Tschesche et al., 1968). The precursor tuliposide A was isolated thereafter as well as tulipalin B and its

precursor tuliposide B. It was first believed that tuliposides store tulipalins for release only after infection or mechanical damage, but it was later shown that free tulipalins also occur at low concentrations in healthy plants. Tuliposide A and tulipalin A have allergenic properties, and are responsible for the skin irritation often suffered by tulip harvesters (called “tulip fingers”) (Hausen et al., 1983; Gette and Marks, 1990). Tulipalin B and the other tuliposides are not allergenic to humans, but these compounds appear to play a protective function to the plant (Beijersbergen and Lemmers, 1972; Schönbeck and Schroeder, 1972).

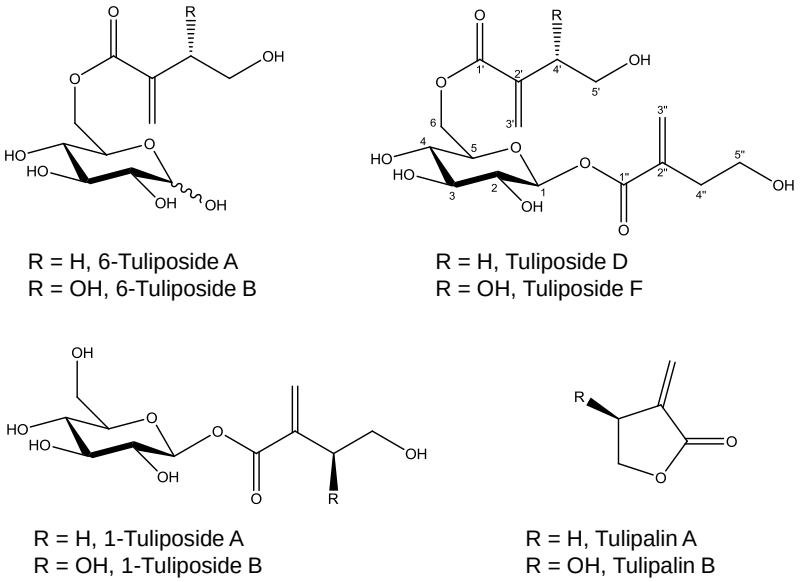

Figure 7. Chemical structures of tuliposides and tulipalins.

Isolated tuliposides and tulipalins have been tested for various bioactivities. As mentioned already, tulipalin A was found to be active against *Fusarium oxysporum* f.sp. *tulipae* (Bergman and Beijersbergen, 1968). The presence of tuliposide A seems to protect against fungal infection of bulbs by *F.oxysporum* and *Botrytis cinerea*, but this may be due to the release of tulipalin A upon infection (Schönbeck and Schroeder, 1972). Both 1-tuliposide A and B were found to have antibacterial properties against *Bacillus subtilis* (Tschesche et al., 1968). Activity against *Pythium debaryanum* was also reported for 1-tuliposide B (Tschesche et al., 1969). In a more recent study, 6-tuliposide B was tested for antimicrobial activity against various bacterial strains. Activity was found in all bacteria tested (including gram-positive and –negative types),

namely *Escherischia coli, Salmonella enteritidis, Pseudomonas aeruginosa, P. glume, P. avenae, Staphylococcus aureus* and *Bacillus subtilis*. Tulipalin B was reported to have antibacterial activity against *E.coli* (Lee et al., 2008). The very effective antimicrobial properties of these compounds are reflected in a patent submitted recently (Park et al., 2008). In addition to the antimicrobial activities of the tulipalins and tuliposides, insecticidal activity has also been reported for tulipalin A against thrips (*Thrips palmi, Frankliniella occidentalis, F.intonsa*) and mite (*Tetranchus urticae*) (Kim et al., 1998).

Gum formation in plants is generally thought to be a defensive response against pathogen infection, to stop their spread and isolate infected tissue (Boothby, 1983). In tulips, microscopic analysis of gums induced by *F. oxysporum* showed the presence of hyphae and spores of the pathogen in the gum (Saniewska et al., 2004). In another study gum induced by *F. oxysporum* stimulated mycelial growth and spore formation of the fungus when added to its culture media (Saniewska, 2002a,b). This effect was seen not only on the specialized tulip pathogen, but also in cultures of *F. oxysporum* f.sp. *callistephi* and *narcissi* (Saniewska, 2002c). From these results it was concluded that the gum does not inhibit the development of the fungus, and that it may stimulate mycelium growth or can be used as a substrate by the fungus (Saniewska et al., 2004).

The discovery of tuliposides in tulip gum raises further questions about the gum's role in the plant's response to pathogen attack. Tulipalin A (Figure 7) is reported to be toxic to *F. oxysporum*, while tuliposide A is not. Neither Tuliposide B nor tulipalin B is toxic to *F. oxysporum narcissi* (Beijersbergen and Lemmers, 1972). *In vitro* studies have reported strong antibacterial activity of 6-tuliposide B and tulipalin B (Beijersbergen and Lemmers, 1972; Lee et al., 2008; Shigetomi et al., 2010). Antifungal activity has also been demonstrated against plant pathogen *Botrytis cinerea*, although the specialized tulip pathogen *Botrytis tulipae* has evolved ways to inactivate the toxic compound (Schönbeck and Schroeder, 1972).
Tuliposides are active against various microorganisms and their function in the plant is believed to be protection against pathogens above and below ground (Beijersbergen and Lemmers, 1972; Schönbeck and Schroeder, 1972). Tuliposides are here reported in tulip gum for the first time. It is possible that they also serve a protective role, with the gum acting as a barrier to pathogens and the tuliposides providing further chemical defense. This barrier may not be effective against *F. oxysporum*, but could help protect against infection by bacteria and other fungi. This may be a case of a specialized fungal strain evolving ways to evade the host plant's defense response, as in *Botrytis tulipae* and tulips (Schönbeck and Schroeder, 1972).
Not all tulip cultivars can be induced to produce gum (Kamerbeek et al., 1971). It is not known whether gummosis occurs in wild species of tulips. The possibility exists that

gummosis is an excessive hyper-sensitive response to ethylene that has been unintentionally selected for in certain cultivars through centuries of breeding. The presence of tuliposides in the gum could then be due to the compounds being in the cells of the tissue where gum formation takes place.

The role of the gum and the metabolites it contains may not be completely understood. The presence of one or two tuliposides in relatively pure form in gum extracts, however, makes this a potentially interesting source of these bioactive compounds. Compared to isolating tuliposides from tulip bulbs or leaves, their isolation from tulip gum presents a much simpler task. There is interest in obtaining tuliposides in pure form from plant material (Damude et al., 2003; Shigetomi et al., 2008). Not only the tuliposides, but also the tulipalins have properties that make them potentially interesting industrial compounds (Pickett and Q. Ye, 2008). Gummosis can be induced in otherwise healthy tulip bulbs by exposing them to ethylene or ethylene-releasing substances (De Munk and Saniewski, 1989). This could potentially be done on large scale for the isolation of tuliposides (and tulipalins) for industrial use.

Studies on gum induction in various tulip cultivars.

Introduction

In ornamental tulip cultivation, infection with the pathogenic fungus *Fusarium oxysporum* f.sp. *tulipae* causes many problems. Infection of the bulbs results in the production of huge amounts of ethylene by the fungus (Saniewska et al., 2005). Ethylene is a plant hormone and its presence in excessive amounts can cause physiological effects such as flower abortion, poor rooting, excessive splitting and gummosis in tulips (Kamerbeek and De Munk, 1976). These problems lead to severe losses of infected bulbs, particularly during storage. Bulbs not directly infected by the pathogenic fungus can also be affected, as the excessive ethylene produced in infected bulbs can cause the previously mentioned effects when released into the storage area.

Gummosis (the accumulation and exudation of gum) does not occur in all tulip cultivars, as they are not all equally sensitive to ethylene. In sensitive cultivars, however, gummosis can be triggered by ethylene concentrations as low as 0.1 ppm. The extent of gummosis has been reported to increase with increasing ethylene concentration, up to about 20 ppm (Kamerbeek et al., 1971). It is also known that in cultivars where gummosis occurs, the sensitivity to ethylene varies over time. Sensitivity to ethylene is reportedly highest in the first four weeks after harvest, after which it decreases (Kamerbeek et al., 1971).

Methyl jasmonate (JA-Me) and related compounds (jasmonates) play an important role in the signal transduction pathway in plants in response to stresses (Koiwa et al., 1997). Jasmonates are known to induce gum formation in various plant species. In tulip stems, jasmonates have been shown to play an essential role for gum formation. JA-Me has been shown to alter sugar metabolism in tulip stems, with some stem carbohydrates likely being channeled to gum formation (Skrzypek et al., 2005). While the exact mechanism of ethylene in stimulating gum formation is not entirely clear, it appears that interactions between ethylene and JA-Me signal transduction pathways occur.

The gum of tulip bulbs was shown to contain the small molecule 6-tuliposide B. This compound and related compounds has some interesting bioactivities and properties, as discussed in the previous section. The fact that this compound occurs in the gum in relatively pure form, means that tulip gum may be a good natural source of 6-tuliposide B for industrial uses. Otherwise healthy tulip bulbs can be induced to produce gum, potentially providing a source of the raw material for tuliposide extraction. In this way gummosis, usually seen as a problem in the ornamental tulip industry, can potentially be turned into a solution for a sustainable supply of a useful bioactive metabolite.

In this study experiments were conducted on tulip bulbs of different cultivars to determine how various factors (ethylene and JA-Me application, temperature, mechanical wounding and time) affect the gum production and tuliposide concentration in the gum. For quantitative analysis of 6-tuliposide B and related compounds in the gum a quantitative ^{1}H NMR method was developed. The aim was to see whether production of tulip gum can be optimized as a potential source 6-tuliposide B.

Materials and methods

Development of qNMR method

Different polar NMR solvents were tested as extraction solvent for tulip gum. Solutions of MeOD, D_2O, KH_2PO_4 buffer (pH 6) and MeOD-Buffer (1:1, v/v), were prepared containing maleic acid as internal standard at 0.1 mg/mL. Tulip gum samples (50 mg of freeze-dried Madame Lefeber gum) were extracted with 1.5 mL of each solvent by vortexing for 20 s, then sonication for 30 minutes. The samples were centrifuged and the supernatant was collected for ^{1}H NMR analysis. To assess the extraction recovery of tuliposide B from the gum, a freeze-dried Madame Lefeber gum sample (56 mg) was extracted three times with 1.5 mL buffer as described above. After each round of extraction the supernatant was collected and measured in ^{1}H NMR. The solubility of tulip gum in buffer was tested by adding 1 mL of buffer to 2, 5 and 12 mg samples of freeze-dried Madame Lefeber gum. The samples were sonicated for 10 minutes,

vortexed and left overnight. The next day samples were inspected to assess dissolution of the gum. The samples were centrifuged, and aliquots of 800 µL were collected for ^{1}H NMR analysis.

Extraction method for ^{1}H NMR quantitation of tuliposide B

Gum samples were freeze-dried and ground to a fine powder with a laboratory blender or by pestle and mortar, depending on the available amount. Five mg of freeze-dried ground gum was weighed into 2 mL microtubes. To each gum sample was added 1 mL of KH_2PO_4 buffer (pH 6) containing 0.01 TMSP and 0.1 mg/mL maleic acid. After vortexing for 30 s and ultrasonicating for 15 min, samples were left overnight in the dark at 4 °C to solubilize. The next day samples were vortexed again, then centrifuged for 5 min at 13 000 rpm. The supernatant (800 µL) was collected and transferred to NMR tubes. Samples were stored at 4 °C until measurement.

NMR measurements

^{1}H NMR spectra were recorded with a Bruker AV 500 spectrometer (Bruker, Karlsruhe, Germany). For each sample 64 scans were recorded using the following parameters: 0.167 Hz/point, pulse width (PW) 4.0 µs and relaxation delay (RD) = 1.5 s. FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to 0.0 ppm. The area under the maleic acid signal at 6.20 ppm was compared to the area under the singlet at 5.98 ppm of tuliposide B, and used to calculate the concentration of tuliposide B in the original gum sample. Two-dimensional J resolved NMR spectra and ^{1}H–^{1}H correlated spectroscopy (COSY) spectra were also recorded.

Bulb treatments and induction of gummosis

Bulbs of *Tulipa gesneriana* L. cv. Apeldoorn, cv. White dream and cv. Madame Lefeber (all sift size 10/12, harvested 12 July 2011) were used in these studies. For ethylene treatments, peeled bulbs were placed in a sealed chamber containing a known amount of ethylene gas, for one to three days. When bulbs were in the chambers for more than one day, the ethylene gas was replenished every 24 hours. Before and after ethylene treatment the bulbs were stored at 20 °C. In some treatments bulbs were mechanically damaged by pricking the bulbs with a push pin (four pricks around thickest part of bulb). Other mechanical damage treatments involved bruising the bulbs by pressing them with a flat hand, cutting the bulb from top to bottom four times (penetrating the first and second outer bulb scales) or cutting the bulbs into four segments (top to bottom). An experiment was carried out with methyl jasmonate (JA-Me) applications in combination with ethylene for gum induction. For these treatments

a special vat with a volume of 3.8 L was used. Ten bulbs were placed in each vat, and JA-Me was applied in one of two ways. The JA-Me was applied as a spray (7.6 or 76 µL in 1 mL water sprayed over the bulbs with a perfume sprayer) or on filter paper (7.6 or 76 µL applied to a piece of filter paper, folded and placed in the vat). The vats were sealed and after sealing ethylene (30 ppm) was added to the vat by means of a syringe through the lid septum. Vats were left for 24 hours at 28 °C, after which they were opened and stored at 20° for five days. Each treatment was carried out with 10 bulbs per category, and NMR measurements were carried out on one sample of each treatment representing the pooled gum.

Results

NMR method development for tuliposide quantitation

^{1}H NMR was found to be a suitable method to analyze the tulip gum extracts. The sharp singlet peaks of 6-tuliposide B in a relatively non-crowded region of the NMR spectrum also make it a suitable method for quantitative NMR analysis. For the development of a quantitative NMR (qNMR) method, an internal standard was needed, as well as a good extraction solvent. An internal standard should ideally give a signal close to the target signal to be used in quantitation. It should also not be overlapped with any other signal in the spectrum (Pauli et al., 2005). The internal standard and target compound should be soluble in the chosen NMR solvent, and the compound of interest should be extracted with a high recovery. The stability of the target compound in the NMR solvent is also important for accurate quantitation. Maleic acid was identified as a good candidate for use as an internal standard, as it gives rise to a singlet in the region of δ 6.20 – δ 6.32 in polar NMR solvents.

Different polar NMR solvents were tested for use as extraction solvent for tulip gum (Figure 8). In all the NMR solvents the maleic acid signals were close to the tuliposide singlet signals, but not overlapping with them or any other signals. Relative to maleic acid, the 6- tuliposide B signals were the most intense in the water and KH_2PO_4 buffer extracts, indicating that these are good extraction solvents for this target compound from the gum matrix. Even though D_2O seemed to extract slightly more 6-tuliposide B than the buffer, it was decided to proceed with the buffer as extraction solvent. It is known that tuliposides are unstable at pH above 7 (Beijersbergen and Lemmers, 1972), so it was decided to use an extraction solvent that controls the pH to keep the extract at a slightly acidic pH.

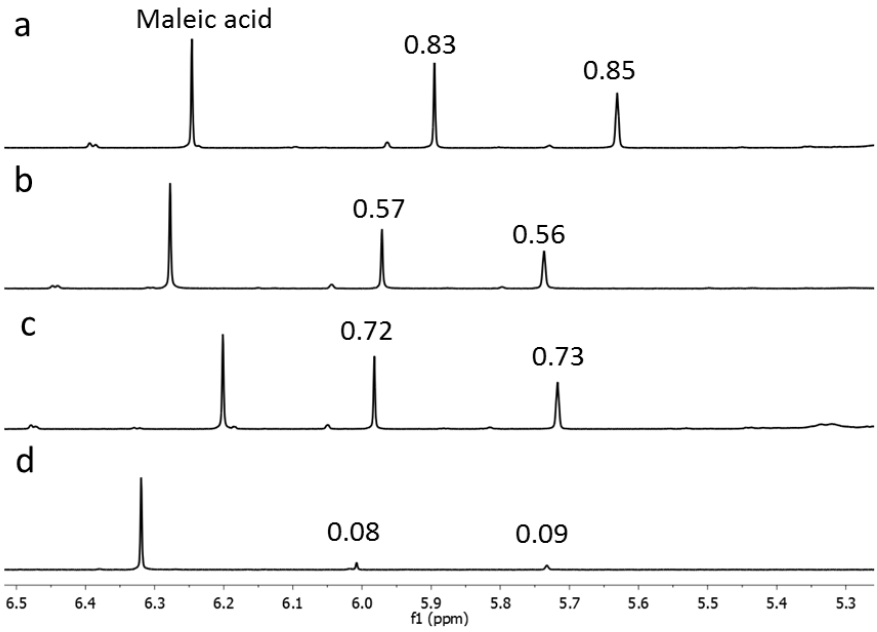

Figure 8. ^{1}H NMR spectra of tulip gum extracted with different polar solvents: (a) D_2O, (b) MeOD-KH_2PO_4 buffer, pH 6.0 (1:1), (c) KH_2PO_4 buffer, pH 6.0, (d) MeOD. The ratio of the 6-tuliposide B singlet signal integrals relative to the maleic acid signals are shown in the Figure.

The extraction recovery of 6-tuliposide B from the gum was tested. It was found that extracting 50 mg of tulip gum in 1.5 mL of KH_2PO_4 buffer (the weight and volume typically used in the NMR metabolomics method) does not give exhaustive extraction of the compound, even after more than three rounds of extraction. This indicated saturation of the solvent, or is related to the fact that the gum is not solubilized completely at these ratios. Smaller volumes of gum were dissolved in buffer to determine how much gum can be completely dissolved in the solvent. Table 2 shows the result of dissolving different amounts of the same gum sample in 1 mL of buffer, and using maleic acid to quantify the amount of 6-tuliposide B in each sample. The gum was completely dissolved when 2 or 5 mg was solubilized in 1 mL of buffer. The amount of 6-tuliposide B also increased proportionally with the amount of gum dissolved up to about 5 mg of gum. Samples prepared with more gum did not dissolve completely and the increase in tuliposide was not exactly proportional to the increase in the amount of gum. Therefore it was decided that 5 mg of gum dissolved per mL of buffer would be optimal for sample preparation.

Table 2. Increase in 6-tuliposide B signal (δ 5.98) in proportion to the amount of gum dissolved in 1 mL of KH_2PO_4 (pH 6.0) buffer.

Gum (mg DW)	Area ratio of 6-tuliposide B singlet to maleic acid singlet
2.0	0.14
5.0	0.36
12.0	0.27

Quantitation of 6-tuliposide B in gum from different cultivars

The presence of 6-tuliposide B was first discovered in gum from cultivar Apeldoorn. The process of gummosis is known to occur in certain tulip cultivars (Kamerbeek & De Munk 1976; Kamerbeek 1971). Bulbs of some cultivars observed to be sensitive to gummosis were obtained and induced to produce gum. The gum was analyzed to determine whether 6-tuliposide B occurs in all the samples. The ^{1}H NMR analyses showed the presence of 6-Tuliposide B in all the gum samples tested (Figure 9). The concentrations ranged from less than 10 mg/g to more than 90 mg/g DW. In cultivars where more than one gum sample was obtained, some variation can be seen between samples (e.g. Madame Lefeber). Since these gum samples were obtained at different times in different conditions, not much can be concluded about the concentrations of 6-tuliposide B in the gum.

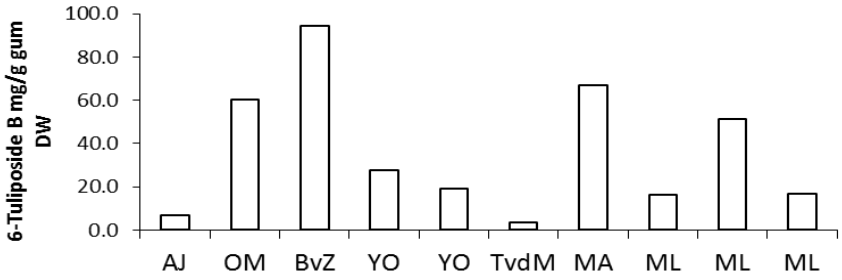

Figure 9. 6-Tuliposide B concentration in gum of different tulip cultivars. AJ: Anna Jose, OM: Orange Monarch, BvZ: Ben van Zanten, YO: Yokohama, TvdM: Tineke van der Meer, ML: Madame Lefeber.

Effect of mechanical injury on gum production and 6-tuliposide B in gum

The effect of mechanical injury on the 6-tuliposide B content in tulip gum in three different cultivars was investigated. Bulbs of cultivars White dream, Madame Lefeber and Apeldoorn were treated with ethylene for 24 hours to induce gummosis. Half the

bulbs of each cultivar were pricked with a needle, and the rest were not damaged. The concentration of 6-tuliposide B was determined and the results are shown in Figure 10. The White dream bulbs produced very little gum, and the tuliposide concentration was not determined in these samples. The Apeldoorn gum had a higher 6-tuliposide B concentration than the Madame Lefeber samples, but less gum was produced by the bulbs. In this experiment mechanical injury with a needle did not have a big effect on the gum production in the Madame Lefeber samples. In the Apeldoorn bulbs the 6-tuliposide B concentration in the damaged gum samples was lower than the control.

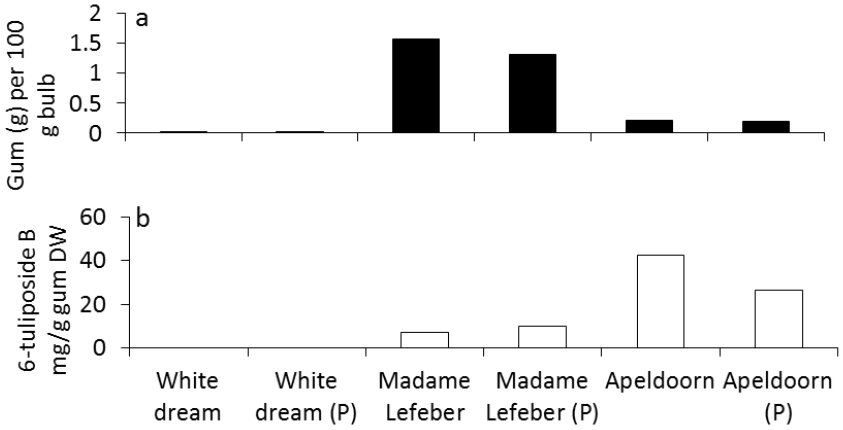

Figure 10. Weight of gum (a) and 6-tuliposide B concentration (b) of gum of three tulip cultivars, treated with ethylene for 24 hours and stored at 20°C for three days. WD: White dream, WD-P: White dream pricked, MD: Madame Lefeber, MD-P: Madame Lefeber pricked, AP: Apeldoorn, AP-P: Apeldoorn pricked.

Effect of duration of ethylene treatment on gum production and 6-tuliposide B in gum

The effect of the duration of ethylene treatments on tuliposide content in the gum was investigated in bulbs of cultivar Apeldoorn, White dream and Madame Lefeber. Bulbs were treated with ethylene for 24 or 48 hours, and then kept for three days at 20°C. Half the bulbs were mechanically damaged by pricking and half were untreated. The results are shown in Figure 11. In all the cultivars, more gum was produced with longer ethylene treatments. Mechanically damaging the bulbs by pricking seemed to enhance gum production in the Apeldoorn and Madame Lefeber bulbs, but not the White dream bulbs. 6-Tuliposide B concentrations were lower in gum of pricked bulbs than untreated bulbs. The undamaged Apeldoorn bulbs treated with ethylene for 24 hours had the

highest concentration of 6-tuliposide B (239 mg/g DW). A single treatment was carried out where bulbs of Apeldoorn were exposed to ethylene for a total of 168 hours. This resulted in the production of 6.48 g gum per 100 g bulbs, the highest amount in any of the experiments performed. The 6-tuliposide B concentration in the gum was relatively low, at 11.8 mg/g gum (DW).

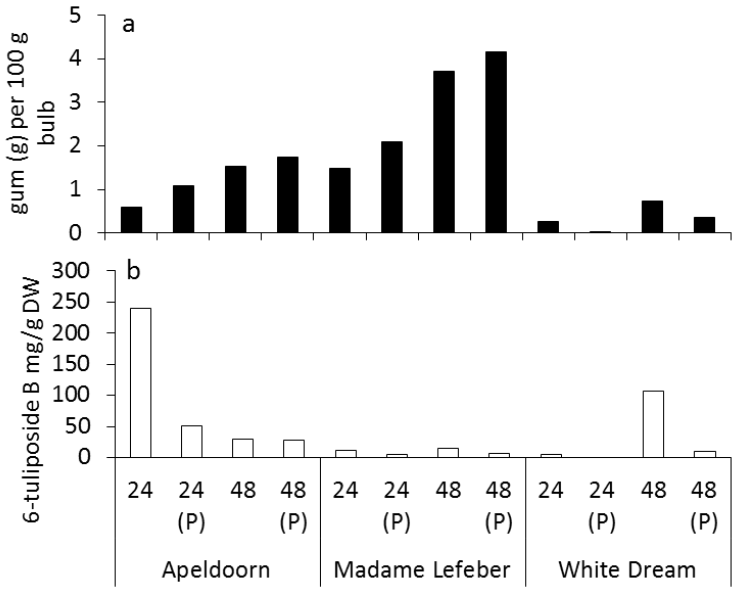

Figure 11. Weight of gum (a) and 6-tuliposide B concentration (b) of gum produced by Apeldoorn, Madame Lefeber and White Dream bulbs, induced with ethylene (Eth) for 24 or 48 hours. Some bulbs were mechanically damaged by pricking with a needle (P).

Effects of different types of mechanical damage on gum production and 6-tuliposide content

Bulbs of cultivar Apeldoorn were given different types of mechanical damage to determine if this had an effect on the content of 6-tuliposide B in the gum, and the amount of gum produced. Bulbs were either pricked with a needle as before, bruised by pressing down on them with a flat object, cut with a knife or quartered. For each treatment, bulbs were treated with ethylene for 24, 48 and 72 hours. The results of the 6-tuliposide B quantitation are shown in Figure 12. From Figure 12a can be seen that the duration of ethylene treatments had a clear effect on the amount of gum produced, with

it increasing with the duration of ethylene treatment. An exception is the quartering treatment, which resulted in very little gum production. The concentrations of 6-tuliposide B were highest in the 24 hour-treated samples in all treatments, and lower in the longer ethylene treatments. The control bulbs not mechanically damaged had the highest 6-tuliposide B concentration in all durations of ethylene treatment.

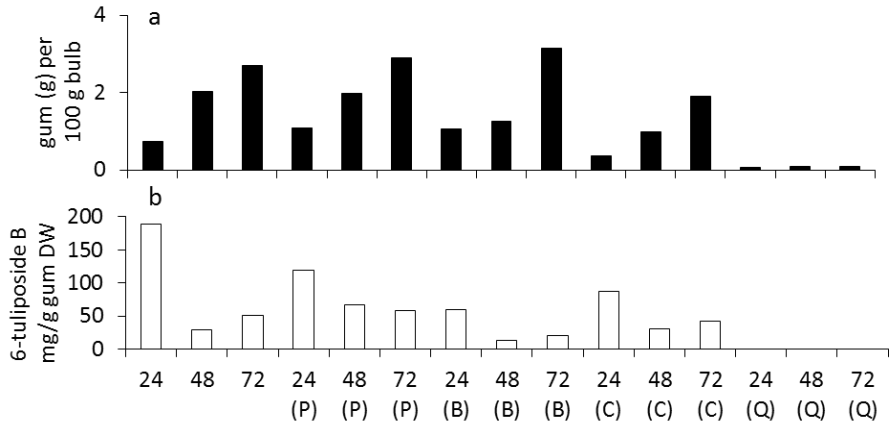

Figure 12. Weight of gum (a) and 6-tuliposide B concentration (b) of gum produced by Apeldoorn bulbs, induced with ethylene for 24, 48 or 72 hours and stored at 20°C for three days. Bulbs were mechanically damaged by pricking with a needle (P), bruising (B), cutting (C) or quartering (Q).

Effect of time after harvest on gum production and tuliposide content of tulip gum

In the previous investigations, there were big differences in the tuliposide content of gum that had been induced in the same way (e.g. 24 hour ethylene treatment, no mechanical damage in Figure 10 and Figure 11). The difference between the bulbs in those treatments was that they had been induced to produce gum at different lengths of time since being harvested. An experiment was performed with Apeldoorn bulbs induced to produce gum at different times after begin lifted from the soil. The bulbs were either damaged by pricking or not, and were treated with ethylene for 24, 48 or 72 hours as before. It was observed that gum extruded to the outside of the bulb and the internal gum between the bulb scales had a slightly different color and texture. In the later stage of this experiment, the external and internal gum was collected and analyzed separately. The results of this investigation are summarized in Table 3.

In this experiment more gum was produced the longer the duration of ethylene treatment at all time points. In the earliest time point (6 days after harvest), the concentration of 6-tuliposide B increased with longer duration of ethylene treatment, but in the following ones the opposite effect was seen. In the later time-points where internal and external gum was collected separately, it is more difficult to see clear trends. Big differences in tuliposide concentrations were seen, however. In most cases the gum extruded externally had higher concentrations of 6-tuliposide B, although this trend was reversed in some samples.

Table 3. 6-Tuliposide B content in gum and weight of gum of Apeldoorn bulbs induced at different times after harvest. Bulbs were treated with 24, 48 or 72 hours of ethylene gas, and half the bulbs were mechanically damaged. In some samples internal (^{i}) and external (^{e}) gum was collected and analyzed separately. Weight is given as sum of internal and external gum weight.

days after harvest	**pricked**	**6-tuliposide B mg/g gum DW**			**gum production g per 100 g bulbs**		
		24 hr	**48 hr**	**72 hr**	**24 hr**	**48 hr**	**72 hr**
6	no	42.69	68.99	-	0.22	1.22	-
6	yes	26.61	60.90	-	0.19	1.25	-
8	no	239.27	29.69	-	0.60	1.53	-
8	yes	51.16	27.25	-	1.08	1.76	-
13	no	189.22	29.63	50.37	0.75	2.03	2.69
13	yes	119.54	67.03	57.56	1.09	1.97	2.88
27/28	no	119.25	21.11^{i}	46.71^{i}	0.41	1.47	2.67
			259.63^{e}	173.74^{e}			
27/28	yes	45.27	188.06^{i}	15.41^{i}	1.18	2.29	4.90
			26.46^{e}	238.61^{e}			
42/44	no	44.00^{i}	291.46^{i}	182.11^{i}	0.49	0.71	1.97
		196.64^{e}	46.96^{e}	63.10^{e}			
42/44	yes	67.09^{i}	44.35^{i}	53.50^{i}	1.13	3.02	3.32
		171.67^{e}	190.36^{e}	178.67^{e}			

Effect of methyl jasmonate treatments on gum production and tuliposide in gum

The effect of methyl jasmonate application on gum production and 6-tuliposide B concentration in the gum was investigated. The experiment was performed at 37 days after harvest of the bulbs. Methyl jasmonate was applied either as a spray or on filter paper to evaporate into the air in the vat. Although the higher concentration JA-Me spray (2000 ppm) treatment resulted in more gum production compared to the others

(Figure 13a), the methyl jasmonate treatments did not seem to make a big difference to the concentration of 6-tuliposide B in the gum (Figure 13b).

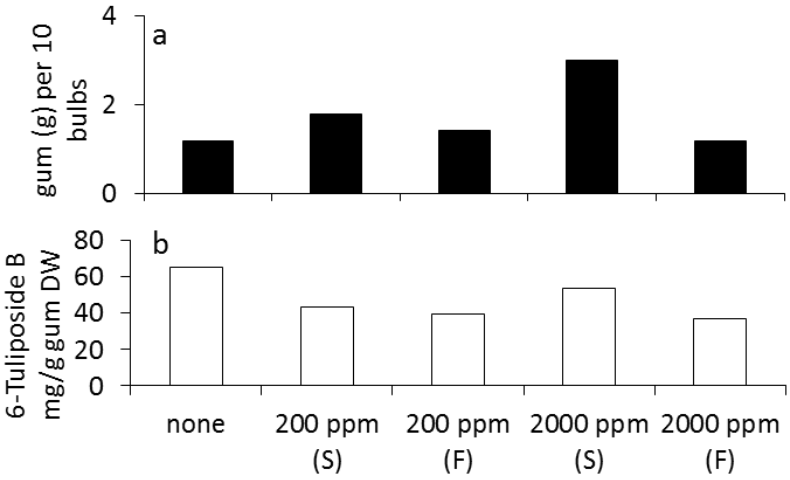

Figure13. Weight of gum (a) and 6-tuliposide B concentration (b) of gum produced by Apeldoorn bulbs induced with 50 ppm ethylene for 24 hours, and different amounts of methyl jasmonate applied either as spray (S) or on filter paper (P). Controls were also treated with 50 ppm ethylene for 24 hours. All samples were treated at 28°C, treatments were done 37 days after harvest.

Discussion

In the process of gummosis gum is made and accumulated in blisters in the tulip bulbs scales. The blisters can burst open so that the gum is extruded, either to the outside of the bulb or into the space between bulb scales. Collection of the gum extruded to the outside is easy, while more effort is required to collect gum between bulb scales and in blisters. In this study the total amount of gum collected per treatment was indicated, but of the total amount sometimes as much as 80% of the mass is from difficult to obtain blisters. Gum produced by cv. Apeldoorn tended to occur more in blisters and between bulb scales, while in cv. Madame Lefeber more gum was extruded to the outside of the bulb. The gum collected in the various treatments varied in texture from softer and gel-like to hard and dry. It was observed that the softer gum, when left exposed to air, would dry out and become hard after a time. All the gum samples were freeze-dried before extraction for NMR analysis to be able to compare 6-tuliposide B content in terms of dry weight. The water content was found to vary between about 7-80% of fresh weight.

As already mentioned, it is known that certain tulip cultivars are more sensitive to ethylene than others. In this study, bulbs of cv. White Dream were the least sensitive to ethylene, and bulbs of cv. Madame Lefeber seemed to be the most sensitive in terms of gum production.

Mechanical damage had previously been observed to increase gum production in certain tulip cultivars (H. Gude personal communication). In this study pricking with a needle was tested as a form of mechanical damage to see if gum production or tuliposide content would be enhanced. In cv. Apeldoorn this mechanical damage had no significant effect on gum production, but the 6-tuliposide B content was lower in gum from damaged samples. No significant effect of needle pricking on gum production or 6-tuliposide B content was seen in gum from cv. Madame Lefeber. When different types of mechanical damage were investigated, none had a positive effect on either gum production or 6-tuliposide B concentration as compared to the control. Cutting the bulbs into four pieces drastically reduced the amount of gum produced.

One effect that can be clearly seen from these experiments is that the duration of exposure to ethylene increases the amount of gum produced. This was the case in all three cultivars investigated. In Apeldoorn the 6-tuliposide B concentration in the gum seemed to follow roughly the opposite trend, decreasing with increased ethylene exposure time. A similar trend seemed to occur in Madame Lefeber samples as well, although this cultivar was not as extensively investigated as cv. Apeldoorn.

In tulip stems, applying JA-Me together with ethylene results in increased gum production (Skrzypek et al., 2005). This enhancement was not seen in our experiment on tulip bulbs. It is possible that the signaling pathways interact differently in the bulbs as compared to the stems. JA-Me applied to the bulbs may be less able to penetrate the cells or to have an effect in this organ.

The absolute values of gum weight and 6-tuliposide B concentration in identical treatments of different experiments were not always the same. For example 6-tuliposide B concentrations in Apeldoorn gum treated for 24 hours with ethylene ranged from about 60-239 mg/g DW in experiments conducted at different times. This is probably related to when the experiments were performed in relation to time of harvest. This has been reported to affect the amount of gum produced in tulips (Kamerbeek et al., 1971). From the results presented here it seems that this also affects the concentration of 6-tuliposide B in the gum.

Induction of gummosis in healthy tulip bulbs was investigated as a potential method of obtaining 6-tuliposide B and related compounds for industrial use. 6-Tuliposide B was

found to be present in all tulip gum samples tested. The production of gum depends on various factors, such as sensitivity of the cultivar to ethylene, duration of exposure to ethylene and time of gummosis induction relative to harvest. With the optimal conditions, it has been shown that gum can be produced from tulip bulbs in this way at amounts more than 6 g gum per 100 g bulbs. As was shown in this study, however, the 6-tuliposide B concentration varies in the gum. It was observed that sometimes the 6-tuliposide B concentration decreased with increased gum production. This suggests that there is a dilution of the small molecule as more gum is produced. It may therefore not be optimal to have long ethylene exposure times to produce large amounts of gum, if the 6-tuliposide B concentration in it will be very low. Depending on the cultivar, gum is mostly accumulated inside blisters and between bulb scales, or otherwise extruded to the outside. For ease of collecting large amounts of gum, cultivars that mostly extrude gum to the outside of the bulb would be better suited for a bigger operation. For truly large-scale operations, a more automatic way of obtaining the gum or the tuliposide from the gum may have to be developed.

This was an initial study to investigate the effects of various induction conditions on the gum production and tuliposide content of the gum. This method has potential for the production of pure 6-tuliposide B from tulips. Production costs would likely be high due to high labor costs, so it would be best suited for the production of the compound for use as a high-value fine chemical. This as opposed to its production as a starting material to make tulipalins for production of bioplastic copolymers, for example, where large amounts of the monomer is needed at low cost. Further studies on structural aspects and properties of the gum itself may reveal uses for the gum as is: a polysaccharide gum containing a small bioactive metabolite. For such use it would be necessary to produce gum with standardized tuliposide content. With careful optimization and control of all conditions involved in producing the gum, this should be possible.

Other bulbs species are also known to produce gum in response to pathogens and/or ethylene, such as species of *Iris* (Liliaceae) and *Hyacinthus* (Hyacinthaceae) (Kamerbeek and De Munk, 1976). Grape hyacinth (*Muscari armeniacum*, Hyacinthaceae) bulbs can be stimulated to produce gum as well (Miyamoto et al., 2010). Gum produced by these and other bulbs may contain interesting small molecules, as these plant families are known to contain many diverse secondary metabolites. The gum may be worth investigating as novel sources of bioactive molecules in relatively pure states.