Assessment of the internal quality of stored flower bulbs using magnetic resonance imaging

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Toetsing van de interne kwaliteit van bloembollen tijdens de bewaring met behulp van magnetische resonantie beeldvorming

(met een samenvatting in het Nederlands)

Proefschrift

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At the peak of a fever like none other,
A good burgher whose thrift was his repute
Might part with two hogsheads of vintage port,
Twelve stout ewes and eight fat swine,
A silver chalice and a suit of clothes,
And brick after wheel after brick of cheese
For a single bulb, and fancy himself shrewd.
The logs disclose another who swapped a mill,
And one a brewery, for their fabled specimens.

Clouds of golden pollen. The pages crackle.
All Holland's in thrall — the tulips have souls.

David Barber

Voor mijn ouders

Contents

Chapter 1

General introduction

Flower bulbs are a major export product of the Netherlands. In 2001, the Netherlands produced an estimated number of 10 billion flower bulbs, which is 65% of the total world production. Tulip, narcissus and lilies are the most important crops. More than half of the flower bulbs is used for the forcing market for cut flowers and pot plants and the rest for the dry sales market for parks and home gardens. More than 75% of the total Dutch production is exported to the United States, Japan and to countries within the European Union. (*Sources: Internationaal Bloembollen Centrum, Productschap Tuinbouw*)

Flower bulbs

Periodicity of flower bulbs

Under natural circumstances, flower bulbs are exposed to seasonal changes in temperature, rainfall and light (Le Nard and De Hertogh, 1993a). The growth cycle of the bulbs is adapted to that periodicity. Depending on the season of flowering, growth activity of the bulbs takes place during spring or autumn. Spring flowering bulbs have a rest period during the summer and resume growth during autumn (Le Nard and De Hertogh, 1993a). They require a warm-cold-warm temperature sequence for growth and development. Typical examples are the tulip, freesia, narcissus and hyacinth. Summer flowering bulbs rest in winter and resume growth in spring. Typical examples of summer flowering bulbs are lily, allium and gladiolus.

The time of flowering is not related to the time of flower initiation. Flower initiation can take place during different periods of the year and at different stages of bulb development. Hartsema (1961) distinguished seven periods of floral initiation, which are depicted in Figure 1.1.

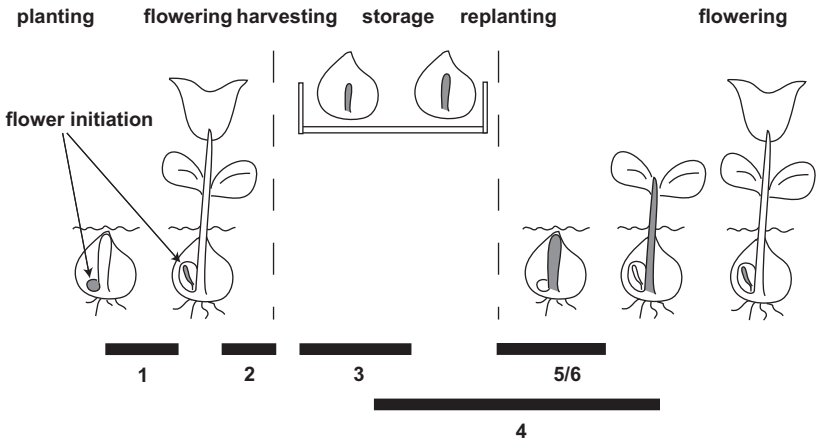

Figure 1.1
Scheme of possible flower initiation/formation periods in flower bulbs according to Hartsema (1961).
1. Flowers are formed more than a year before flowering (e.g. certain amaryllis and nerine species)
2. Flowers are formed almost a year before flowering, just after the previous flowering period (e.g. narcissus)
3. Flower formation occurs after harvest and during storage (e.g. tulip, crocus and hyacinth)
4. Flower formation starts during or towards the end of the storage period, but has to be completed after planting (e.g. lily, dahlia)
5. Flowers are formed after replanting at low temperatures (e.g. iris)
6. Flowers are formed after replanting in spring (e.g. anemone, freesia and gladiolus)
7. Flower and leaf formation alternate and are not dependent on temperature periodicity (e.g. *Hippeastrum* (amaryllis)) (not shown)

Forcing

From the previous, it may be concluded that most flower bulbs have a life cycle of one year or longer, either flowering in spring or in summer. Nevertheless year-round production of cut flowers has become common practice. For the control of flowering, needed for the year-round production of flowers, bulbs are subjected to specific temperature regimes to affect their growth and development. The use of artificial growth conditions for the flowering of bulbs, to simulate those required by nature, is called "forcing" (Le Nard and De Hertogh, 1993a).

Forcing of flower bulbs starts at harvest during the rest period. The applied temperatures during the phase of enlargement affect the physiological state of the bulb at harvest. However, the temperatures in this period can hardly be controlled since it usually occurs outside on the field. Elevated temperatures applied directly after harvesting are used to control the time and rate of flower formation (Le Nard and De Hertogh, 1993a). Subsequently, low temperatures are applied to differentiate the flower bud and initiate rooting. The applied low temperatures are usually necessary to break the rest period or the so-called dormancy of flower bulbs, which is defined as the state of a healthy bulb, characterized by little or no external growth of the shoot and roots (Borochov *et al.*, 1997; Le Nard and De Hertogh, 1993a). After the requirements to break dormancy are fulfilled, bulbs are planted and transferred to a rooting room (to promote root development) or directly to the greenhouse. Depending on the chosen temperature regime flowering can be promoted, retarded or prevented.

Disorders in flower bulb development

Detailed knowledge of bulb periodicity is important for the control of flowering. When forcing conditions are not properly chosen, physiological disorders in bulb development may occur. The use of incorrect temperature regimes and light conditions during storage and planting often result in leaf aberrations and bud abortion (De Hertogh and Le Nard, 1993b). Furthermore, exposure to ethylene during storage or planting can induce disorders like gummosis, necrosis, flower abscission and abortion (Kamerbeek and De Munk, 1976). Flower bud abortion is a large problem in the forcing of flower bulbs and thus an important subject of this study.

De Hertogh and Le Nard (1993b) defined flower abortion as: "the failure of a bulb to produce a marketable flower after the floral organs have been formed". Bud abortion can emerge after planting in multiple forms, ranging from the absence of a flower ('blindness') to the bleaching and withering of the bud. Unfortunately, the effects of the applied forcing conditions and thus the induction of physiological disorders are usually not visible externally. Sometimes, the presence of the disorders can be determined by cutting the

bulb shortly before planting, but in practice the effects become apparent at flowering. When more knowledge is gained about the processes underlying physiological aberrations like bud abortion, their development might become detectable at an early stage during storage. This would avoid the further costs of storage and planting and it potentially increases the chances to minimize the damage. Knowledge of the physiology of flower bulbs and the availability of a tool to detect distorted developments, will also enable the optimization of forcing conditions of new ornamental flower bulb genera. Finally, with the development of a sensitive detector for aberrations, quality can be better guaranteed.

The aim of this study was therefore to gain a better insight in the processes underlying physiological disorders in flower bulbs induced by storage conditions, and to search for tools to assess their internal quality.

The present study of the physiological disorders in flower bulbs is focused on the effect of storage temperatures on the two most important crops in bulb breeding: tulip and lily. Tulip and lily are also typical examples of spring and summer flowering bulbs, respectively.

Tulip

Morphology of the tulip

The morphology of a tulip bulb is depicted in Figure 1.2. A tulip bulb usually consists of two to six fleshy scales, enclosing the shoot. The scales are enlarged

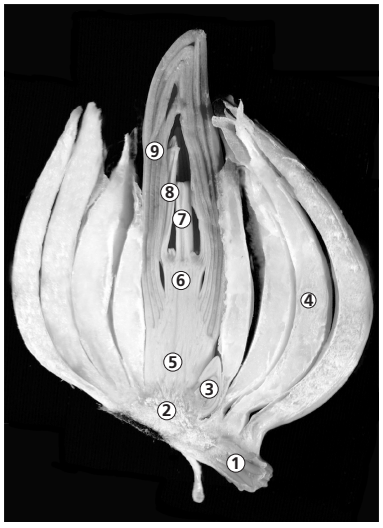

Figure 1.2
Longitudinal section of a tulip bulb cv. Apeldoorn. The tunic has been removed. 1. site of root primordia 2. basal plate 3. daughter bulb 4. scale 5. first internode of the stem 6. upper internode of the stem ('neck') 7. pistil 8. stamen 9. leaves.

leaves and serve as the primary storage organs. The outer dry and brown scale is called the tunic and protects the bulbs from infections and mechanical damage. The basal plate is the short fleshy stem axis of the bulb (De Hertogh and Le Nard, 1993a) from which the shoot and roots develop. The basal plate comprises a network of vascular bundles connecting the scales and roots to the shoot.

Daughter bulbs are developing in the axils of the scales. Growth of the daughter bulbs occurs mainly during planting. They require a minimal size to be able to flower after planting; otherwise they produce a leaf (Le Nard and De Hertogh, 1993b).

Forcing of tulips

For the year-round production of tulip bulbs and cut flowers, different forcing programs are nowadays used in the Netherlands. The bulbs can be left uncooled after harvest (in June/July) and planted outdoors or in the greenhouse for flowering around April, according to the natural periodicity.

To promote flowering, bulbs are dry-stored 1-2 weeks at moderate (20 °C) or high (30-35 °C) temperatures directly after harvest. Subsequently, the bulbs are transferred to low temperatures (2-9 °C) and dry-stored, stored on trays or on water for a certain period. Hereafter the bulbs are transferred to a rooting room or directly to the greenhouse (17-20 °C). This way, the first flowers can be obtained in November. When the storage temperatures remain higher or when the transfer from high to low temperatures is delayed, flowering is also delayed.

To retard flowering to summer or fall, bulbs are frozen stored (so-called ice-tulips). To obtain these ice-tulips, bulbs are stored until November at moderate temperatures (17-20 °C). Subsequently, the bulbs are either planted in trays for three to six weeks at 9 or 5 °C, to enable rooting, or packed in soil after the cold period. Hereafter, bulbs are frozen at -1.5 to -2 °C. After the summer period the trays are placed in the greenhouse or outside and flowers are obtained in autumn. However, this procedure has not yielded good flower quality, so far.

Physiology of the tulip

After harvest and during subsequent storage of the bulbs, flower bud and root differentiation are the primary processes occurring. In June/July, a few weeks after harvesting, the flower bud has reached flower stage G (Cremer *et al.*, 1974). This means that all floral organs have been formed, i.e. two whorls of three tepals and three stamens, and a three-lobed pistil. The differentiation processes of the bud and roots as well as the development of the differentiated

organs are greatly influenced by post-harvest storage temperatures. High temperatures (30-35 °C) are applied directly after harvest to promote floral and root organogenesis and differentiation of the axillary buds (Hoogeterp, 1973; Koster, 1981). Prolonged storage at moderate or high temperatures results in slow growth and finally in desiccation of the bud (Le Nard and De Hertogh, 1993b). Storage at low temperatures (2-10 °C) fastens growth and results in early flowering with longer stems after planting (Le Nard and De Hertogh, 1993b; Moe and Wickstrøm, 1973). When the cold storage is prolonged, growth is more rapid. Further prolongation finally results in short flower stalks with aborted buds and in the growth of daughter bulbs (Le Nard and De Hertogh, 1993b). When the low-temperature storage is started soon after harvest, flower bud differentiation is reduced and after planting it can result in an aborted shoot without root elongation (De Munk and Hoogeterp, 1975). Therefore, tulip bulbs are exposed to a moderate or high temperature after harvesting, whereupon low temperatures are applied.

A cold treatment of sufficient duration after floral organogenesis is necessary to allow a subsequent rapid stem elongation and flower maturation (cold requirement) (Le Nard and De Hertogh, 1993b; Thompson and Rutherford, 1977). For example, *Tulipa gesneriana* L. cv. Apeldoorn requires a minimum of 12 weeks at 5 °C (Boonekamp *et al.*, 1990; Moe and Wickstrøm, 1973). Many studies point to the prominent role of hormones in the breaking of dormancy and the subsequent plant growth (Boonekamp, 1997; Saniewski and Kawa-Miszczak, 1992; Saniewski and Kawa-Miszczak, 1992). It has been suggested that gibberellins and cytokinins induce flower development (Franssen *et al.*, 1997). The flower bud and especially the pistil produce auxin, which controls the elongation of the stem (Banasik and Saniewski, 1979; De Munk, 1979; Suh, 1997). During the cold treatment sensitivity to auxin is increased (Rietveld *et al.*, 2000). In addition, a distribution of carbohydrates from the scale to the flower bud takes place. In the scale, starch is converted into sucrose and fructans (Davies and Kempton, 1975; Moe and Wickstrøm, 1973; Thompson and Rutherford, 1977), after which sucrose is transported to the shoot (Hobson and Davies, 1977) and reconverted into starch (Haaland and Wickstrøm, 1975; Moe and Wickstrøm, 1979). Low temperatures activate carbohydrate metabolism possibly via hormonal control (Moe and Wickstrøm, 1973; Moe and Wickstrøm, 1979). Auxin might increase the sink strength of the flower bud compared to that of the daughter bulbs (Moe, 1979; Saniewski and Kawa-Miszczak, 1992). The processes underlying the cold requirement of tulip bulbs remain intangible.

Figure 1.3
An aborted flower bud from a tulip bulb after planting.

Disorders in tulip bulb development

The application of artificial growing conditions to control the flowering of tulip bulbs for the year-round production of flowers can induce physiological disorders, which eventually reduce flower quality after planting. A few examples of physiological disorders in tulip bulbs are given below.

- Stem topple is induced by a local calcium deficiency resulting from rapid elongation at high forcing temperatures, poor rooting or high relative humidity causing deficiencies in water transport. The top part of the stem becomes dark green and watery and finally shrinks causing the above-lying stem to topple (Nelson and Niedziela Jr, 1998).
- Veinal streak emerges as twisted leaves with discolored leaf veins and, in a severe stage, with glassy, withered patches on the leaf. This disorder may result from wet and cold conditions during rooting (*Source: Internationaal Bloembollen Centrum*).
- Hollow stems occur when a tear develops lengthways in the stem due to rapid development. Excessive water absorption and limited evaporation in the greenhouse are probably at the basis of this disorder (*Source: Internationaal Bloembollen Centrum*).
- Bud abortion manifests with a dry necrotic flower (Figure 1.3). This disorder has numerous causes and can be induced during storage as well as in the greenhouse. Bud abortion is a major problem in tulip breeding.

According to Le Nard and De Hertogh (1993b) tulip bulbs are susceptible to bud abortion when:

1. Low temperatures are applied too soon after organogenesis;
2. The roots do not emerge rapidly after planting due to bruising, a very hard tunic, diseases, excessive or insufficient water, delayed planting, high planting temperatures or because the root system is insufficiently developed;
3. Daughter bulbs are developed before rooting at the expense of the shoot;
4. The planted bulbs are too small. Small sized bulbs are more susceptible to bud abortion compared to large bulbs (De Munk and Hoogeterp, 1975).

Bud abortion can also be induced by exposure to ethylene, for instance from *Fusarium* infected bulbs. Sensitivity to ethylene is maximal directly after harvest and during planting (Kanneworff and Van der Plas, 1994). Exposure to ethylene in combination with mites can lead to bud necrosis. The whole shoot and inner scales become necrotic, resulting in brownish-black colored and wet tissue (De Munk, 1972; Kamerbeek and De Munk, 1976). De Munk (1973) has described the stages of bud blasting (abortion) after exposure to ethylene: 1. The tips of one of the stamens is less rigid; 2. All stamens are flabby; 3. All stamens, filaments and perianth leaves shrivel; 4. The pistil is withering and all floral organs turn yellow; 5. All floral organs are wilted and yellowish brown; 6. The innermost leaf of the bud is wilted; 7. All leaves are wilted, papery and bleached.

After planting of bulbs in stage 1, only the stamens are blasted. When damage is advanced, green streaks and white tips on the perianth are found. In a further stage, small flowers with green and twisted perianth leaves appear. The upper internode will not elongate and the upper leaf will not unfold in case of severe damage (stage 4 to 7).

The time of abortion of a flower-bud can be determined by measuring the length of the bud. If it does not exceed a length of 1 cm, the bud is usually aborted before planting. With longer buds, abortion usually occurred in the greenhouse (De Munk and Hoogeterp, 1975).

Calcium deficiency is assumed to be one of the processes resulting in bud abortion and is related to an impaired water transport through the bulb (Klougart, 1980; Nelson and Niedziela Jr, 1998). The presence of the roots and the leaves are important for bud development and stem elongation, whereas the role of the roots and the leaves can be substituted by gibberellins (Kawa-Miszczak *et al.*, 1992) and IAA or NAA (Saniewski and Kawa-Miszczak, 1992), respectively. Moe (1979) and De Munk and Gijzenberg (1977) suggested

that flower bud blasting is the result of a lack of substrate supply to the bud. The distribution of substrate within the bulb might be controlled by the hormonal status. Gibberellins and cytokinins could prevent ethylene-induced blasting by strengthening the sink of the flower bud (De Munk and Gijzenberg, 1977).

To gain a better understanding of the processes underlying the physiological disorders induced by storage of flower bulbs, bud abortion in tulip bulbs has been studied. Although, bud abortion can be induced by (small) impairments in the hormonal or metabolic activities, the obvious and final result is the dehydration of the flower. Thus in the processes leading to bud abortion, the water status must have changed. By examining the water status of tulip bulbs in which bud abortion was induced, insight in which processes are leading to bud abortion can be increased. Further knowledge on the timing of these processes is also important for the development of a detection tool for the presence of the disorder.

Lily

Lily classification

The *Lilium* family can be subdivided into three classes: *Lilium longiflorum*, *Lilium* Asiatic hybrids and *Lilium* Oriental hybrids. The Asiatic and Oriental hybrids originate from several different *Lilium* species. The three classes of

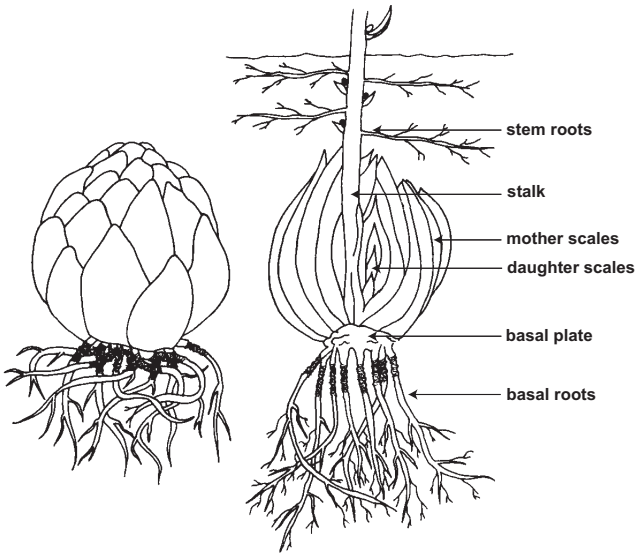

Figure 1.4
Drawing of a lily bulb

bulbs have different flower characteristics. The flowers of *L. longiflorum* (Easter lily) are usually white colored, the flowers of the Asiatic hybrids are usually orange, red, sometimes yellow and white, while the Oriental flowers have more red, pink and white colors. The Oriental flowers have the strongest fragrance (Wilkins and Dole, 1997). The Oriental hybrids are late flowering, while the Asiatic bulbs have a range of flowering times. Focus will be mainly on Oriental hybrids.

Morphology of the lily

Lilium bulbs are white to yellowish non-tunicated bulbs comprised of numerous scales, which surround the shoot imbricately (Figure 1.4). Different generations of tissue can usually be recognized within a lily bulb. The outer scales originate from the 'mother' bulb and are connected to the old bulb basis. On top of the bulb basis lies the new basal plate connecting the shoot to the 'daughter' scales. After planting, roots are developed from the basal plate and the stem. The lily plant usually has a long stem with many leaves and several flowers, comprised of three sepals and three petals, six yellow orange anthers and a three-lobed stigma (Miller, 1993).

Forcing of lilies

Harvest and storage of lily bulbs

In the Northern hemisphere, bulbs are harvested from late August to December. At harvest, Oriental hybrids are dormant (Gude *et al.*, 2000b). Dormancy in lily bulbs seems to be related to high levels of the hormone abscisic acid (ABA) and a low respiratory activity (Gude *et al.*, 2000b).

After harvest, Oriental hybrids are kept at 2 to 4 °C for at least 8 weeks prior to greenhouse forcing (Wilkins and Dole, 1997). To enable growth and development in spring, a period of low temperatures after harvest is required to break dormancy and to decrease ABA levels (Gude *et al.*, 2000a). Depending on the temperatures in the soil, the breaking of dormancy can also be initiated in the soil prior to harvest (Schouten *et al.*, 1997).

When the bulbs are to be stored for longer periods, temperatures are further reduced to -1 to -4 °C. In this way the bulbs can be stored year-round (Wilkins and Dole, 1997). When storage is continued at temperatures just above 0 °C, the shoot continues to grow out of the bulb and becomes sensitive to mechanical damage, which can cause a decrease in flower quality (Boontjes, 1983). At freezing temperatures, growth of the shoot is arrested. During storage at frost temperatures, actual freezing of the bulbs does not occur (Miller and Langhans, 1990). Lily bulbs are stored in moist peat, especially when kept at freezing temperatures to prevent dehydration (Beattie and White, 1993; Hartsema, 1961).

The sugar content inside the bulb increases during the period of cold after harvest (Kok *et al.*, 2001) probably due to the conversion of starch in the scales, which is enhanced at low temperatures. Yet, this increased sugar content can also be obtained in the soil, when harvest is delayed and temperatures are sufficiently low. During storage, the sugar content rises to a maximum value and remains at that level for a few weeks upon which it decreases again (Kok *et al.*, 2001). Frozen storage should be started at the time point of maximal sugar content. The maximum sugar content varies between cultivars and years and determines the frost tolerance of the bulbs. Asiatic hybrids generally have a higher maximal sugar content and thus can be stored at lower temperatures (-2 °C) compared to Orientals (-1.5 °C) and *Longiflorums* (-1 °C) (Kok *et al.*, 2001).

Flowering of lily bulbs

The time and development of flower initiation varies greatly between *Lilium* species and can be divided into subclasses (Wilkins and Dole, 1997):

1 Flowers initiate in late summer and are well developed by autumn (comparable to period 2 in Figure 1.1);
2 Flower initiation commences in late summer but the development is not completed until the following spring (comparable to period 4 in Figure 1.1);
3 Flowers initiate and develop in spring, prior to shoot emergence (comparable to the beginning of period 5 in Figure 1.1);
4 Flowers initiate in spring after shoot emergence (comparable to the end of period 5 in Figure 1.1).

Oriental hybrids can be categorized into most of these subclasses.

Flowering conditions in the greenhouse are very critical during forcing of lily bulbs. Day and night temperatures, irradiation and photoperiod have large effects on the flower quality and time of flowering. Long photoperiods enhance floral initiation (Wilkins and Dole, 1997). Increasing irradiation increases the rate of flower development and the number of flowers formed but leads to a decreased plant height (Wilkins and Dole, 1997). The (difference between) day and night temperatures also influences the plant height, the number of days to flowering (Erwin and Heins, 1990; Roh and Wilkins, 1973) and the number of abnormal flowers (Lee and Roh, 2001).

Disorders in lily bulb development

During forcing of lily bulbs, the plant quality can be diminished by:

- Bud abortion, which is considered to be initiated at the beginning of bud

development and manifests as a small white dot in the axils of the bracts (Roh, 1990a).
- Bud blasting, which is characterized by bleaching of the green color, beginning at the base of the bud. Ultimately, the bulbs show a brownish necrosis, become desiccated and eventually fall off, leaving the pistil behind (Durieux *et al.*, 1983).
- Bud abscission, which is characterized by a similar bleaching of the bud followed by the development of a constriction at the pedicel-bud junction and rapid shedding of the bud (Durieux *et al.*, 1983).
- Leaf scorch, which appears with greenish-yellow to white spots, results from a calcium deficiency caused by an unbalanced water status. Large bulbs are more susceptible to this (*Source: Internationaal Bloembollen Centrum*).
- Frost injuries, which can range from aberrations in the leaf development to short plants without flowers (Schouten *et al.*, 1997).

Flower-bud blasting and flower-bud abscission occur when light intensities are low, i.e. during forcing in wintertime. These aberrations can be avoided with additional lighting (Durieux, 1975; Van Tuyl and Kwakkenbos, 1986). Flower-bud abscission (and bud blasting) was found to be related to an increased ethylene production of the flower bud (Beattie and White, 1993; Mason and Miller, 1991; Van Meeteren and De Proft, 1982). Water deficiency and carbohydrate depletion are also mentioned in relation to bud abscission and bud blasting in lilies (Roh and Wilkins, 1973; Roh, 1990b; Van der Meulen-Muisers and Van Oeveren, 1996). Early flowering cultivars produce more blasted buds due to long-term frozen storage, whereas late flowering cultivars produce more aborted buds (Roh, 1990a). Factors affecting bud abortion have hardly been addressed. The symptoms are very small and can be easily overlooked.

In the present study the injuries caused by frozen storage for year-round production of flowers from Asiatic and Oriental hybrids are further examined. Frost injuries first manifest in the leaves at the lower part of the stem, which become laced up, while the flowers develop normally. In more severe cases, the shoot apex becomes injured resulting in a small plant without flowers. In the worst case, the shoot does not emerge above the ground (Schouten *et al.*, 1997). Frost injuries can occur when the frozen storage is started too soon or too late after harvest (Boontjes, 1983), when the sugar content was not yet maximal or decreasing again (Boontjes, 1981; Kok *et al.*, 2001). Frost injuries can also be induced when the applied frost temperatures are too low. Unfortunately, the frost tolerance is variable per cultivar and year (Gude *et al.*, 2000a).

Frost damage is probably a result of desiccation stress (Burke *et al.*, 1976). The formation of ice-crystals results in the withdrawal of water from the cells to balance the water potentials (Steponkus, 1984). Thus frost injuries probably affect the water status within the bulb.

Detection of floral aberrations

The detection of floral aberrations in flower bulbs at an early stage of development can increase the physiological understanding of the underlying processes and reduce the costs involving further storage and forcing. Indicators for flower quality can also be used for the optimization of the storage protocols. Especially when the floral aberrations involve changes in the water status, for example in case of dehydration, magnetic resonance imaging (MRI) is a logical research tool. The principles of MRI are explained in the addendum.

Magnetic resonance imaging

MRI can measure the presence and physical properties of water molecules in tissues, non-invasively. Thus, time-dependent changes in morphology and the water status can be assessed without cutting the bulb. Morphological changes can be detected on the basis of the presence of MR-signal, which is most easily done by T_2-weighted imaging. Multiple water status parameters can be obtained from the same bulb, each providing different information.

- The maximal intensity of the MR-signal is related to the amount of protons present and can thus be used to determine the local water concentration.
- T_1 and T_2 relaxation times can give information on the motional properties of water protons. In free, mobile water, T_1 and T_2 relaxation times are long, but when water is interacting with components like proteins and other macromolecular structures or when the solution is viscous due to high sugar concentrations, the relaxation times are shortened. Thus, changes in the environment of water molecules can be reflected in changes in T_1 and T_2 relaxation times. T_1 relaxation is often related to the water content (Ratcliffe, 1994; Ruan and Chen, 1998). T_2 is more sensitive to diffusion processes through cellular compartments with different constituents and thus with different relaxation characteristics (Hills and Nott, 1999).
- With the use of pulsed field gradients, nuclei can be magnetically labeled and the displacement of the labeled nuclei can be followed. The velocity and potential anisotropy of diffusion (ADC) and flow processes can thus be determined.

- The magnetic interactions of water with immobile protons, like in starch and proteins can be studied by Magnetic Transfer (MT) experiments. By applying a long irradiation pulse, off-resonance with respect to the free water peak, magnetic interactions between the immobilized protons and the free water pool can be visualized (Bendel *et al.*, 2001). Double quantum-filtered measurements focus on the water pool that is restricted in mobility in a certain orientation (Bendel *et al.*, 2001).
- Water protons form the major contributor to the measured ^{1}H-NMR signal. However, protons can also be found in for example carbohydrates, but these signals are usually overshadowed by the bulk signal of water. The water signal can be suppressed, making discrimination of the MR signals of less abundant components possible. Changes in the levels of these substances can also be studied locally. Apart from ^{1}H, other nuclei can also be studied, including ^{31}P, which allows the study of the energy status via the levels of high-energy phosphates. These techniques as a group are referred to as magnetic resonance spectroscopy (MRS).

MRI and floral aberrations

Because of the possibilities of MRI for studying the water status of plant material, it is expected that it can provide information on the processes underlying storage-induced physiological aberrations in flower bulbs. Based on the work of other research groups it was anticipated that the water status in flower bulbs can be assessed (Bendel *et al.*, 2001; Iwaya-Inoue *et al.*, 1996; Okubo *et al.*, 1997; Van der Toorn *et al.*, 2000; Yamazaki *et al.*, 1995; Zemah *et al.*, 1999). Most research has been done with respect to the cold requirement of bulbs. Comparison of the reported result is difficult because experiments have been done on different flower bulbs, under different conditions and with the use of different parameters. Yamazaki and coworkers (1995) were the first to rapport on MRI and flower bulbs. They measured T_1 values in allium bulbs at 270 MHz and found an average T_1 over the whole bulb decreasing from 1.77 s to 0.8 s when harvesting time was delayed coinciding with increased amounts of fructans in the bulb. Research on allium was also reported by Zemah *et al.* (1999). They measured a (T_1-weighted apparent) T_2, based on two different echo times at 200 MHz and found significant changes in the values of the basal plate as a result of vernalization.

MRI has further been used to study tulip bulbs. Iwaya-Inoue *et al.* (1996) studied T_1 relaxation in chilled and non-chilled bulbs. In the cortex of the scales they found a T_1 of 0.8 s in chilled bulbs and a T_1 of 0.5 s in non-chilled bulbs at 270 MHz. They related the increased water mobility to the presence of metabolically active cells. Okubo *et al.* (1997) found a lower T_1 value in

the second scale after precooling (0.7 s at 270 MHz) compared to the control, non-cooled bulbs (1 s).

Van der Toorn *et al.* (2000) did a more extensive survey on the water status in tulip bulbs. They reported on T_1, T_2, proton density and the apparent diffusion coefficient (ADC) at low magnetic field (20 MHz). They found lower values in T_1, T_2 and ADC in cold-stored (4 °C) bulbs compared to control (20 °C stored) bulbs. The authors suggested that T_1 and T_2 are correlated to the free water content, while the ADC offers a more direct measure of water mobility.

The most direct comparison of the consequences of the use of different magnetic fields can be made on basis of the data reported by Bendel *et al.* (2001) and Van der Toorn *et al.* (2000). At the start of storage Van der Toorn *et al.* (2000) found at 20 MHz, a T_1 and T_2 of 0.58 s and 118 ms, respectively. Bendel *et al.* (2001) determined the T_1 and T_2 at 200 MHz at the beginning of storage in one bulb. T_1 and T_2 amounted to 0.56 s and 29 ms, respectively. As result of the higher magnetic field, T_2 was altered most, while, as Van der Toorn *et al.* (2000) indeed indicated, T_1 is most dependent on the applied magnetic field. Bendel and co-workers (2001) found that, as a result of chilling, the immobile proton pool as well as the pool of orientation restricted water molecules was decreased. They suggested that chilling of bulbs enhances metabolic processes in the scales, leading to the conversion of bound/ordered to free water. These data imply that the processes during the cold fulfillment result in changes in the water status, which can be sensitively detected by MRI.

From the previous it may be deducted that the biological interpretation of the acquirable MRI parameters is not always straightforward. In addition most parameters can not be determined independently of each other. For example, if transverse (T_2) relaxation in flower bulbs is fast, it will influence signal intensities in many other measurements.

In this study the use of MRI is restricted to the measurement of T_1 and T_2 relaxation times and water concentration values at one magnetic field strength (4.7 Tesla, 200 MHz). For the determination of the T_2, a multislice spin-echo sequence was used. Technical limitations excluded the implementation of a reliable multi-echo sequence, suitable for the measurement of short T_2 components. T_1 relaxation times are determined by an inversion recovery spin-echo sequence, which is twice as sensitive as saturation recovery sequences.

Alternative methods

Although MRI is a very sophisticated technique for visualizing water in plant tissue non-invasively, the biological implications of the obtained (relaxation)

data for the water status in plants is not so evident. In addition to MRI parameters, classical water status parameters were obtained from flower bulbs. The combined techniques are used to clarify the processes underlying the physiological developments. Classical methods may reveal the processes governing the T_1 and T_2 relaxation. The classical approach involved for example the determination of the water content by fresh and dry weights. The water content can indicate net water loss as well as water displacements in the tissue and is often assumed to affect longitudinal relaxation times (Ruan and Chen, 1998). Aside from the water content, the osmotic potential, water potential and the ion-leakage from tissues were determined.

Outline of this thesis

The purpose of the research described in this thesis was to study physiological disorders in flower bulbs induced by sub-optimal storage conditions. This was done with the use of MRI combined, with classical water status measurements. Furthermore, it was assessed whether these parameters might serve as early indicators of impaired flower bulb quality.

Bud abortion in tulip bulbs, induced by long-term cold and dry storage is discussed in Chapter 2 and 3. In Chapter 2, MRI was used to follow the development of a number of tulip bulbs as a function of storage time. Water content, osmolality and ion-leakage values were obtained additionally. In Chapter 3, this study was extended for two additional seasons, except that the bulbs were imaged first and subsequently planted. Aside from T_1 and T_2 relaxation, non-invasive water concentration measurements were included. The latter was first tested on potato tubers. Water potential and microscopy data were added to gain a better understanding of the processes involved in bud abortion. In the addendum to Chapter 3, the biological meaning of the relaxation times is discussed in more detail.

To study whether the water status in flower bulbs as a result of bud abortion, is altered according to general principles, bud abortion was also induced by storage at high temperatures (Chapter 4). The water status was studied by MRI and classical water status parameters. To assess the year-to-year variability, experiments were carried out in two consecutive years.

Studies on freezing injury in (Oriental hybrid) lily bulbs, induced in two consecutive years, are described in Chapter 5. Freezing injury was induced by freezing bulbs more deeply and by delayed application of the usual freezing temperatures. In the addendum to Chapter 5 the characteristics of water-soaked lily bulbs are discussed.

Examples of the physiological disorders in flower bulbs (tulip, lily and *Hippeastrum*), that are detectable by MRI are presented in Chapter 6.

In summary, in this study flower bulbs were exposed to sub-optimal storage conditions like too long dry-storage (in tulip), storage at too high temperatures (in tulip) and storage at too low temperatures (lily). The effects of these storage conditions on the development of flower bulbs are discussed in the general discussion (Chapter 7). In this chapter the feasibility to apply MRI in horticultural practice is also discussed.

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Addendum to chapter 1

Principles of magnetic resonance imaging

(Source: Ruan RR, Chen PL. 1998. Water in foods and biological material; a nuclear magnetic resonance approach. Lancaster: Technomic Publishing Company, Inc.)

Any nucleus with a non-zero spin quantum number ($I \neq 0$), when placed in a magnetic field, can absorb and emit energy through electromagnetic radiation, which can be detected by NMR. The ^{1}H nucleus, the proton, is by far the most commonly used by NMR, because it can be sensitively determined (high γ) and it is abundantly present in most biological tissues. Besides ^{1}H, also ^{13}C, ^{17}O, ^{19}F, ^{23}N and ^{31}P are used in biological and medical studies.

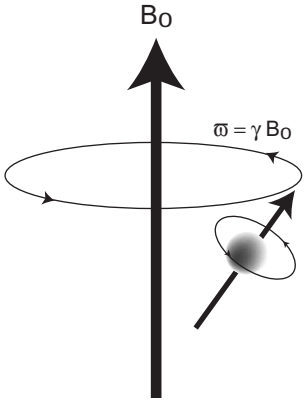

Figure 1.5
The magnetic moment of a nucleus precessing about the static magnetic field B_0.

A nucleus with I≠0 is spinning around its axis and is generating a small magnetic field (Figure 1.5). When this nucleus is placed in a static magnetic field it aligns its magnetic field parallel or anti-parallel to the static one. It will start precessing about the static magnetic field. The frequency of the precession is called the Larmor frequency (ω) and is dependent on the strength of the static magnetic field (B_0) and the gyromagnetic ratio (γ), as indicated by equation 1.

Equation 1: $\omega = \gamma B_0$

The gyromagnetic ratio is nucleus dependent and amounts for a proton 42.6 MHz/Tesla. When B_0 is 4.7 Tesla, protons will precess with a frequency of 200 MHz.

Dependent on the direction of the magnetic field of the nucleus compared to the static magnetic field (parallel or anti-parallel), the nucleus is in a higher or lower energy level. The lower energy level (parallel to B_0) is somewhat more favorable and slightly more nuclei will be in this state. Thus, the net magnetization of all nuclei is oriented along the direction of the B_0 field and amounts M_0, which is the maximal value (Figure 1.6A).

The B_0 field is traditionally defined to be aligned along the z-axis. The different nuclei precess in the x,y plane. It is often convenient to define, next to the laboratory frame, a second frame of reference (rotating), which rotates

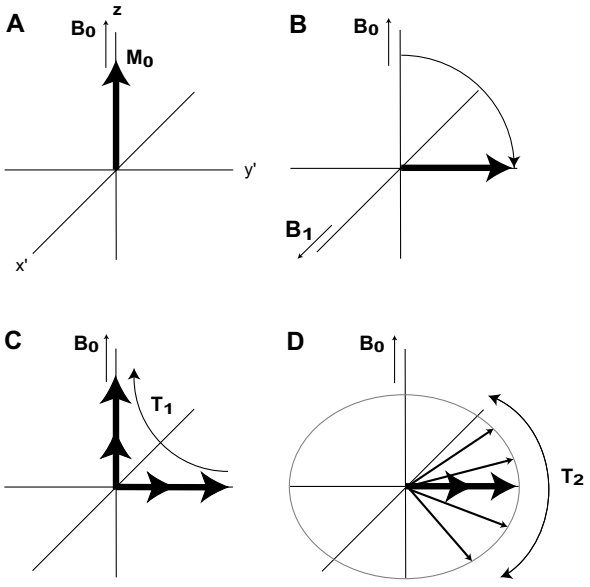

Figure 1.6
The net magnetization of all the nuclei is oriented along the direction of the B_0 field and amounts to M_0 (A). A 90° RF-pulse (generating a B_1 field) along the x'-axis will rotate the net magnetization vector into the x',y'-plane and along the y'-axis (B). After the application of the RF-pulse the net magnetization will return to thermal equilibrium along the z-axis due to T_1 relaxation (C) and dephase due to T_2 relaxation (D).

at the Larmor frequency. The rotating frame, in which the nuclei seem to stand still, is described by the x',y',z-axis.

When a second magnetic field (B_1) is applied perpendicular to the B_0 field, which is rotating at a frequency matching the Larmor frequency, transitions of the nuclei from the lower energy level, and visa versa, are induced. By the application of a Radio Frequency (RF) pulse, which generates the B_1 field, the net magnetization vector can be flipped by an angle α from the z-axis. For example, a 90° pulse will bring the net magnetization vector entirely in the x',y' plane (Figure 1.6B). After the application of the RF pulse, the net magnetization will return to thermal equilibrium along the z-axis due to two relaxation processes. Due to longitudinal relaxation processes the nuclei will return to the lower energy level and thus to the parallel direction (Figure 1.6C). The energy is transferred to surrounding nuclei, which precess at the same frequency. The relaxation time constant T_1 describes this relaxation process. The longitudinal relaxation process is faster (T_1 shorter) when energy transfer is more efficient due to the presence of more surrounding nuclei with the same Lamor frequency. Since the Lamor frequency is proportional to B_0, T_1 will vary as a function of B_0. The second relaxation process, transverse relaxation, is described by T_2. Due to transverse relaxation processes, the nuclei will lose their phase coherence, which leads to progressive dimming of the MR signal (Figure1.6D).

Directly after a 90° RF pulse, the signal will be maximal whereupon it will decrease to zero due to longitudinal and transverse relaxation processes. This signal is called a free induction decay (FID). Because the static magnetic field is not perfectly homogeneous, nuclei in different parts of the sample experience slightly different magnetic fields and thus precess at slightly different frequencies. This results in a faster decrease of the magnetization in the x'y'-plane. This problem can be overcome with the use of a spin-echo

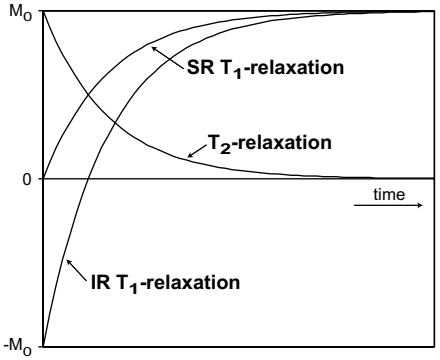

Figure 1.7
Time-dependence of signal evolution in T_1 and T_2 measurements. The intensity of the NMR signal as a function of echo-time (TE) in a T_2 measurement. The intensity of the NMR signal as a function of inversion or recovery-time in inversion recovery (IR) or saturation recovery (SR) T_1 measurement, respectively.

sequence. Following the 90° pulse, a 180° pulse is applied after some time τ. During τ, phase coherence is lost due transverse relaxation and field inhomogeneities. By application of the 180° pulse, the magnetization is refocused and peaks at time 2τ. By increasing τ (or echo time TE), signal loss is primarily governed by transverse relaxation. The transverse relaxation process can thus be sampled by measuring at different τ's (Figure 1.7). The system is left to return to equilibrium before each new pulse sequence of a 90° and 180° pulse is applied. To accelerate the measurements, multiple 180° pulses can be applied after a single 90° pulse. This is called a multi-echo sequence.

Longitudinal relaxation can be determined in two ways. After a 90° and 180° pulse sequence no magnetization is left in the z-direction. Due to longitudinal relaxation, magnetization in the z-direction increases. When applying a 90° and 180° pulse sequence before equilibrium was reached, only that part of the signal that had recovered is measured. Thus when the delay time is zero, no signal will be measured, when the delay is longer than the time needed for equilibration, the signal will be maximal. By changing the delay time, the longitudinal relaxation can be assessed (Figure 1.7). This method is called saturation recovery. When an inversion recovery method is used a 180° inversion pulse precedes the normal 90° and 180° sequence. Due to the 180° inversion pulse the magnetization is aligned to the -z-axis. The magnetization recovers hereafter in the z-direction. The amount of recovered signal is determined by the timing of the subsequent 90° and 180° pulse-element. By varying the time after inversion, the inversion time, the longitudinal relaxation time can be quantified (Figure 1.7).

The longitudinal relaxation time T_1 and transverse relaxation time T_2 are influenced by the chemical and physical environment of the measured nuclei. Thus, nuclei in different or changing environments can be discriminated by NMR.

By applying a one-dimensional linear magnetic field gradient in combination with a frequency selective RF pulse, only the nuclei at a chosen position along the gradient direction will be excited. This corresponds to the excitation of a slice through the object with a certain slice thickness. Subsequently, by application of magnetic field gradients in the two perpendicular directions, the magnetization of nuclei in the slice can be spatially encoded. From the MR signal, a two-dimensional projection of the sample can be derived by Fourier transformation. This results in an MR-image, build up of image-elements, each containing information on the proton density, relaxation characteristics and a range of additional parameters, depending on the pulse sequence used.

Chapter 2

Bud abortion in tulip bulbs studied by magnetic resonance imaging

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Abstract

After storage and subsequent planting of flower bulbs the flower bud frequently appears to be aborted. This physiological aberration is probably caused by a change in the water status of the bulb and may be initiated during storage. The development of bud abortion in tulip bulbs was studied during long-term dry storage of the bulbs at 5 °C. The anatomy of individual tulip bulbs was followed non-invasively with T_2-weighted NMR imaging, which allowed the monitoring of the growth of the shoot and daughter bulbs. Quantitative maps of T_1 and T_2 relaxation times of individual bulbs were used to assess regional changes in the water status of different tissues. Parallel to the NMR measurements, bulbs were planted to assess the ultimate flower quality. Moreover, water content, osmolality of tissue sap and ion leakage of excised shoot and scale tissues were determined to obtain information about the water status and viability of the bulbs. Significant decreases during long-term storage were found in T_1 and T_2 relaxation times in the shoot and particularly in the stamens. An increase in the osmolality of tissue sap and the decrease in relaxation times in the shoot below a certain threshold value attained after 24 weeks of storage, could be indicative for the emergence of bud abortion in tulips.

Introduction

In horticultural practice, bud abortion (blasting), i.e. ceasing of the development of the shoot in flower bulbs, is a substantial problem in several crops, including hippeastrum, nerine and tulip. The cause and course of this physiological disorder are unknown. In addition, its presence can only be visibly detected shortly before, or after planting. To assess the development of bud abortion in bulbs, the tulip bulb is an attractive model system. The physiology of tulip bulbs has been studied widely in the past and bud abortion can be induced in tulip bulbs during storage. Moreover, failures in tulip forcing are often due to abortion of the flower-bud, which may occur during various phases of development (De Munk and Hoogeterp, 1975). Bud abortion can be induced by long, cold dry storage (Le Nard and De Hertogh, 1993), storage at high temperatures (De Munk and Hoogeterp, 1975; Rees, 1973) or by exposure to ethylene (De Munk, 1973; De Munk and Hoogeterp, 1975).

The physiological disorder is a result of the cessation of the development of the flower bud and may manifest itself in tulips with a short upper internode of the stem, also termed 'neck', with an approximately 1 cm long bud with papery white tepals (De Munk, 1973; De Munk and Hoogeterp, 1975; De Munk and Gijzenberg, 1977) and yellow-brownish stamens. Flower-bud abortion has been attributed to a lack of substrate supply to the bud (De Munk and Gijzenberg, 1977; Moe, 1979) or an impaired hormonal activity (De Munk and Gijzenberg, 1977). Nevertheless, desiccation of the tepals and stamens and a deficient stem elongation are expected to be the final result of a change in the water status.

NMR-imaging (MRI) enables non-invasive, longitudinal assessment of the local water status of plant tissues (Donker *et al.*, 1997; Kuchenbrod *et al.*, 1995; Millard *et al.*, 1995). It has been used to study several physiological disorders in fruits and vegetables (Chudek and Hunter, 1997; Clark *et al.*, 1997; Faust *et al.*, 1997). Therefore, MRI might be useful to study the water status of flower bulbs, which could give insight into the development of the bulbs and related disorders like bud abortion. The magnetic properties of water, which can be measured by MRI, include the longitudinal and transverse relaxation times (T_1 and T_2, respectively). In plant studies T_1 and T_2 are often used as markers for the water status as they are primarily correlated with the water content and the mobility of water (Ratcliffe, 1994; Ruan and Chen, 1998).

Tulip bulbs (cv. Apeldoorn) require 12 weeks of storage at 5 °C to obtain proper growth and development of the shoot (Boonekamp *et al.*, 1990; Moe and Wickstrøm, 1973). This cold requirement for tulip bulbs has been studied frequently (Kanneworff and Van der Plas, 1994; Lambrechts *et al.*, 1994;

Rietveld *et al.*, 2000) also with the use of MRI (Bendel *et al.*, 2001; Iwaya-Inoue *et al.*, 1996; Okubo *et al.*, 1997; Van der Toorn *et al.*, 2000). However, the process of bud abortion has not been studied by means of NMR techniques.

When dry-stored at 5 °C for a period longer than approximately 20 weeks the flower within the tulip bulb starts to abort (Le Nard and De Hertogh, 1993). In the present study long-term storage was used to induce bud abortion. T_2-weighted images were obtained at several time points during the storage period to monitor changes in bulb morphology and anatomy. Furthermore, T_1 and T_2 relaxation time maps were calculated to monitor longitudinal changes in the water status during the same period. The relaxation times of the scale and the whole shoot and of specific areas within the shoot were analyzed in order to evaluate developmental processes at tissue level.

To obtain information about the emergence of flower bud abortion, bulbs were planted parallel to the NMR measurements. Water content and the osmolality of tissue sap of bulbs were determined during the storage period to obtain information on the physiological processes influencing the water status. Ion leakage experiments were carried out to test for viability of the tissue (Bonnier *et al.*, 1992; Bonnier *et al.*, 1994).

The present study aimed to investigate whether MRI, in combination with other (classic) assays of tissue water status, can give an insight into the process of flower abortion in tulip bulbs. The ultimate goal of these experiments is to identify a parameter, which is indicative of the initiation of bud abortion at an early stage of its development.

Materials and methods

Plant material

Tulip bulbs (*Tulipa gesneriana* L., cv. Apeldoorn), 12-13 cm in circumference, were harvested in July 1998, and stored at 20 °C until two weeks after flower differentiation stage G (Rees, 1973). Subsequently, the bulbs were dry-stored at 5 °C and 70-90% relative humidity, for 34 weeks.

NMR imaging

NMR images were obtained on a 200 MHz NMR instrument (Varian, Palo Alto, CA) interfaced to a 4.7 T, 40 cm horizontal bore magnet. A 85 mm Helmholtz volume coil was used. Images were measured with a multislice spin echo sequence, using a Field of View (FOV) of 6x6 cm^2; 256x256 matrix (0.2x0.2 mm^2 in plane resolution); 2.5 mm slice thickness; 5 slices chosen such that the middle slice intersected the bud; 2 transients were averaged.

T_2-weighted images were measured with the following parameter set: echo times (TE) of 5, 7, 10, 15, and 20 ms; repetition time (TR) of 5 s, to calculate T_2 maps. T_1-weighted images were obtained with the inversion recovery technique using inversion times (TI) of 0.001, 0.2, 0.5, 1, and 5 s; TE of 5 ms; TR of 5 to 10 s.

T_1- and T_2-weighted images of 5 bulbs were collected over a period of 34 weeks. After 12 weeks of storage three of these bulbs were planted to determine the quality of the flowers. Three other bulbs were measured after 13 weeks of storage and followed throughout the rest of the protocol. To minimize discontinuities in the storage conditions, NMR measurements were done at 5 °C by blowing cold air via a heat exchanger into the cylinder in which the bulbs were fixed.

Data processing

T_1 and T_2 maps were obtained by mono-exponential fitting of the T_1- and T_2-weighted images on a pixel-by-pixel basis using a program written in Interactive Data Language (IDL).

Regions-of-Interest (ROI's) as chosen in the five slices are depicted for the central slice in Figure 2.1 and include an area in the second outermost scale, the whole shoot, an area in the basal plate, two regions in the stem (first and upper internode) and a region including the stamens and pistil. ROI's were placed in the T_2-images with the shortest TE, which have the highest signal intensity. T_1 as well as T_2 values of the calculated maps were averaged for each ROI and averaged over the relevant slices through the bulb.

Assessment of flower quality

Bulbs were planted after 12 to 28 weeks of dry storage at 5 °C. At each time point 20 bulbs were potted and grown in a ventilated room at 17 °C, 75% RH and with a 16 hr light period (200 μmol m^{-2} s^{-1}). Tulips with a bud of approximately 1 cm length, with papery white tepals, and a short upper internode of the stem were designated as aborted. Flowers, which did not have completely red colored tepals and did not open completely, were designated aberrant.

Water content

The shoot and three punches of tissue (7 mm diameter) taken from the second outermost scale were weighed and dried for at least 4 weeks at 70 °C. The second outermost scale was used in this investigation because the first scale was often (mechanically) damaged. The water content of scale and bud tissue was calculated from the difference between fresh and dry weight and expressed

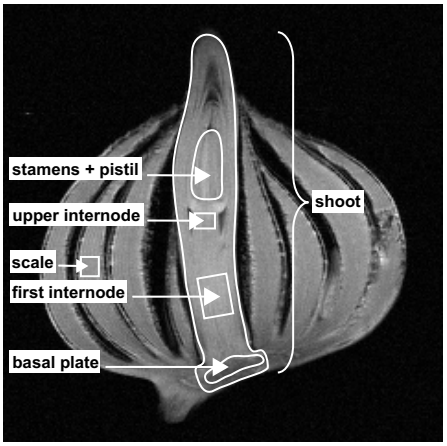


Figure 2.1
Regions-of-Interest in the slices (shown for the central slice), which were chosen to evaluate the NMR relaxation parameters during storage in five bulbs.

on a fresh weight basis. The water content of normal and aborted flowers after planting (at anthesis) was also determined.

Ion leakage

A disc (13 mm diameter) from the second outermost scale was punched out, weighed and incubated in 50 mL distilled water per gram tissue. After 24 hrs of incubation at 20 °C the conductivity of the incubation media was determined with a Radiometer Copenhagen conductivity meter. To determine the total amount of ions present, the tissue samples were boiled for 1 hr and the conductivity of the medium was measured again at 20 °C. The same procedure was followed for the whole shoot (until 12 weeks of storage) and for the shoot, divided into two parts i.e. the basal plate plus first internode of the stem and the bud (from 12 to 34 weeks of storage). At every time point during storage ten bulbs were dissected and measured. A reference curve was obtained with KCl solutions.

Osmolality of tissue sap

Pieces (of approximately 1 g) of the second outermost scale and shoot of 10 bulbs were stored at -20 °C for 2 hrs. After 14 weeks of storage the shoot was divided into three parts, i.e., the basal plate plus first internode of the stem, the second until fifth internode and the floral tissue (bud). After thawing, the tissue was placed in a tube with a pin on top to squash the tissue during 10 min of centrifugation at 1000xg. Subsequently, the supernatant was centrifuged for 5 min at 15800xg. The osmolality (in mmol kg^{-1}) of 10 µL of the supernatant was determined with a thermocouple psychrometer (Wescor Vapory Pressure Osmometer), a technique based on a dew point measurement.

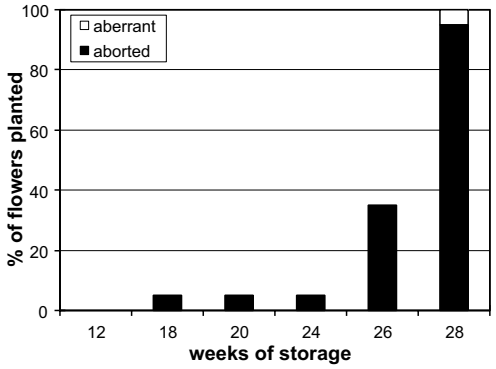


Figure 2.2
Bud abortion after storage and subsequent planting.

Results

Flower development

Flower development was normal after 12 weeks of dry storage at 5 °C and subsequent planting. None of the flowers appeared to be aborted (Figure 2.2). Flower abortion was apparent to a small extent after 18 to 24 weeks of storage increasing steadily beyond this to 35% after 26 weeks and to 95% after 28 weeks of storage. Storage longer than 28 weeks resulted in abortion of the shoot directly after planting. In these bulbs the shoot failed to emerge after planting.

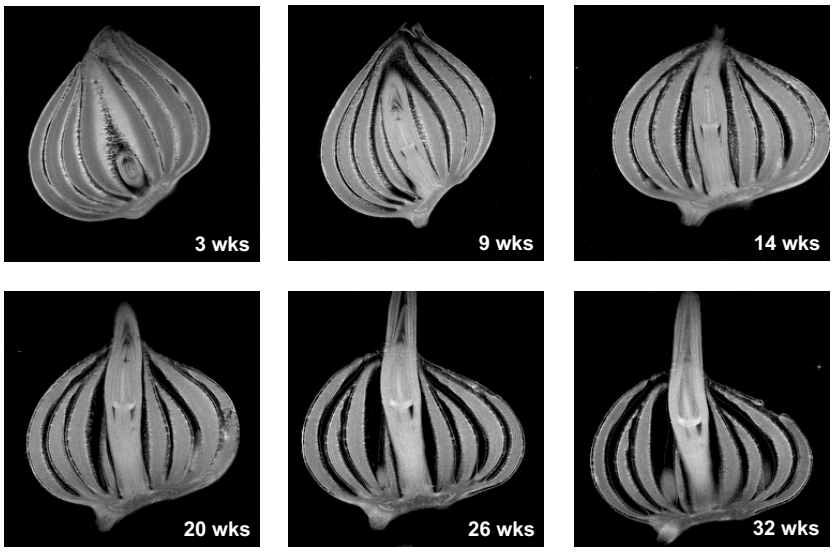


Figure 2.3
Development of a tulip bulb during 32 weeks of dry storage at 5 °C, monitored by T_2-weighted imaging (TE=0.005 s, TR=5 s). A single slice through the center of the shoot of the same bulb is shown.

NMR measurements

Anatomy

NMR-images of one and the same bulb monitored during 34 weeks of dry storage at 5 °C are shown in Figure 2.3. The shriveling and separation of the scales in time is very clear as well as the presence of bruises in the scale tissue. Furthermore, shoot development in time is evident and seems normal. The stamens, pistil, upper internode of the stem and vascular tissue, especially in the basal plate, can be distinguished. In Figure 2.3 a daughter bulb is visible after 26 weeks of storage but in most bulbs the appearance and growth of daughter bulbs were observed after approximately 14 weeks.

T_1 relaxation times

Quantitative T_1 and T_2 maps of the five bulbs were calculated from series of T_1- and T_2-weighted images. Typical examples are shown in Figure 2.4. Within the T_1 maps different areas within the scales can be distinguished: the central, (sub)epidermal and in between the 'lateral' area. The central part of the scale showed a lower T_1 value compared to the lateral tissue (0.45 s vs. 0.52 s at the beginning of storage) until approximately 12 weeks of storage (quantitative data not shown). The (sub)epidermal layer appeared to have a very long T_1.

To follow the development of the individual bulbs, average T_1 and T_2 values of the ROI's of the five measured slices were calculated for each bulb as a function of storage time (Figure 2.5). The ROI in the second scale used

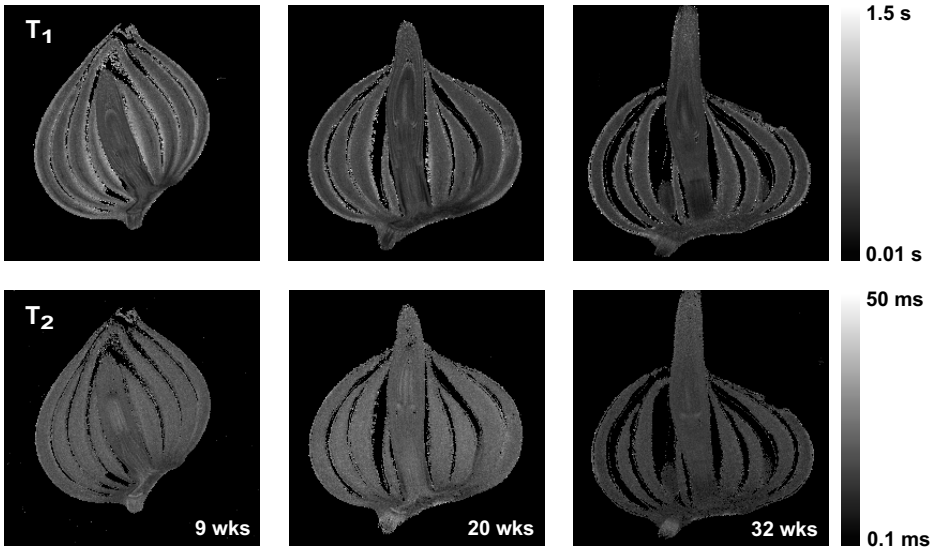
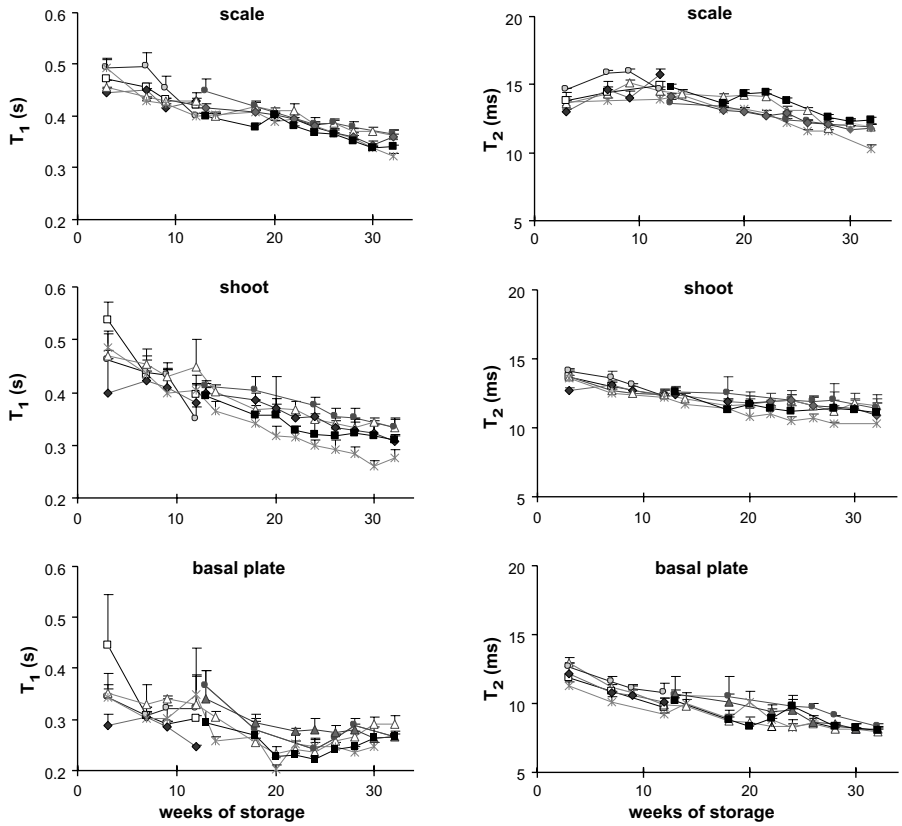


Figure 2.4
T_1 (upper row) and T_2 maps (lower row) of the bulb shown in Figure 2.3, as collected during storage. Bright areas represent long relaxation times, dark areas short times.

for Figure 2.5 included the central as well as lateral tissue but not the (sub)epidermal tissue. A decrease in T_1 values of the ROI of the second scale was observed in all bulbs during storage. The decrease was mainly found in the lateral tissue of the scale. After approximately 12 weeks of storage the T_1 relaxation time of the lateral tissue decreased to similar values as for the central part making distinction of both tissues impossible.

The average T_1 value of the whole shoot also decreased throughout the storage period. However, when focusing on different areas within the shoot, various trends could be distinguished. The T_1 of the basal plate tissue displayed a minimum at 20 weeks of storage and slightly increased thereafter. The T_1 values in the first internode were more variable, but the changes in the values through storage time were similar. The T_1 values started to decrease after 12 to 18 weeks of storage. The T_1 values of the upper internode as well as the stamens region showed a pronounced decrease throughout the storage period, similar to those in the entire shoot.



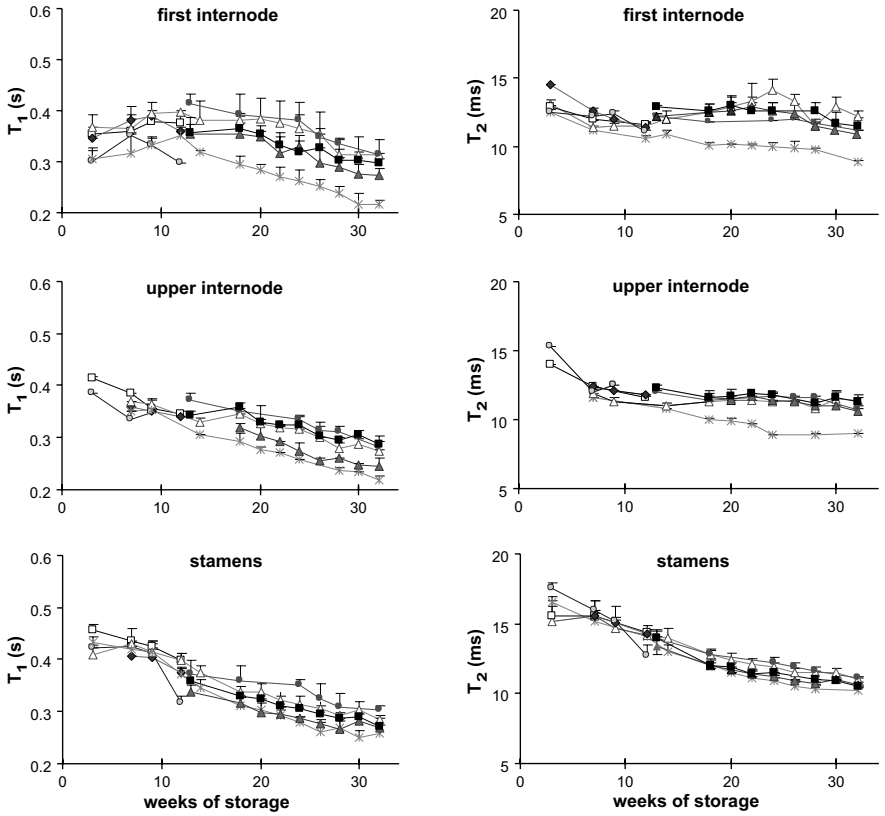


Figure 2.5
Mean T_1 (left column) and T_2 values (right column) of ROI's within the second outermost scale, whole shoot, basal plate, first internode, upper internode and stamens plus pistil area of five bulbs determined as a function of storage time. Error bars represent standard deviations. For further details see Materials and methods.

T_2 relaxation times

In the second outermost scale, the mean T_2 value started to decrease after 9 weeks of storage (Figure 2.5). Within the scale a distinction between central and lateral tissue was not possible on basis of T_2 relaxation values. The average T_2 value in the whole shoot decreased considerably until 18 weeks whereupon the decrease diminished. The average values of T_2 relaxation times in the basal plate decreased during development. In the ROI in the first internode, every bulb showed a different value and pattern in time. A distinct trend in T_2 relaxation times throughout development was not observed in the region of the first internode. In the region of the upper internode a major decrease occurred during the first 7 weeks of storage. However, after 3 weeks only two

shoots were sufficiently large to distinguish the upper internode. From 12 weeks until the end of storage the T_2 relaxation times within the ROI of the upper internode were essentially constant except for one bulb that exhibited a further decrease in T_2. The mean T_2 values in the stamens and pistil area decreased strongly (34%) during storage.

Water content and growth of the shoot

During the first 18 weeks of storage the water content of the second scale (Figure 2.6) hardly changed because the dry matter and amount of water of the punches decreased to the same extent (data not shown). The decrease in water content in the scale after that period was the result of a further decrease in the amount of water while the dry matter mass remained the same. The shoot increased in mass and length throughout the storage period. Until approximately 14 weeks the water content of the shoot decreased as a result of the larger increase in dry matter compared to the amount of water per shoot. After anthesis the water content of normal flowers was 88.6% ± 1.1 (n=34), while aborted flowers only contained 48.0% ± 5.4 (n=17) water.

Ion leakage

The ion leakage from the scale, measured as the conductivity of the medium after 24 hrs of incubation, increased during the first 12 weeks of storage and then reached a plateau at approximately 130 μS cm^{-1} (Figure 2.7). By contrast, ion leakage from shoot tissue decreased until 12 weeks of storage to a value of approximately 16 μS cm^{-1} and then remained constant.

The total amount of ions in both tissues was constant during storage whereas the conductivities of the solutions after boiling remained approximately 220 μS cm^{-1} for scale tissue and 260 μS cm^{-1} for shoot tissue. This corresponds to 85 μmol "KCl-equivalents" per g tissue and 101 μmol "KCl-equivalents" per g tissue, respectively.

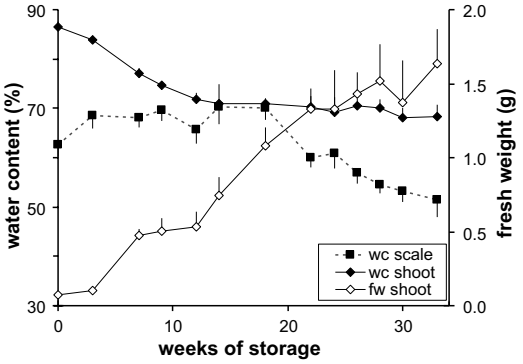


Figure 2.6
Water content (wc) of the second outermost scale and the shoot, and the fresh weight of the shoot (fw), indicating the growth of the shoot as a function of storage time. Error bars represent standard deviations.

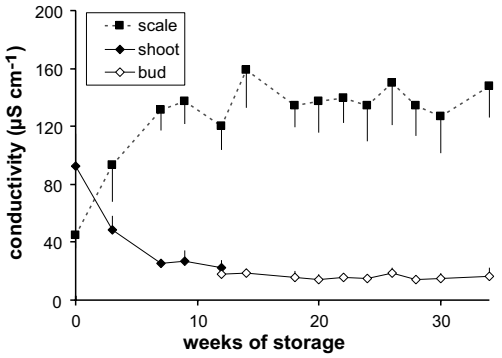


Figure 2.7
Ion leakage from excised tissue of the second outermost scale and the shoot measured as the conductivity of the incubation medium as a function of storage time. The bud contained the floral tissue above the first internode of the stem. Error bars represent standard deviations.

Osmolality of tissue sap

The osmolality of tissue sap, which is a measure for the concentration of total solutes, increased considerably during the first 12 weeks of storage in scale as well as in shoot tissue (Figure 2.8). Between 12 and 26 weeks of storage the osmolality values remained the same whereupon they started to increase again both in shoot and in scale tissue.

Discussion

As was expected for cv. Apeldoorn, growth and shoot development after planting were normal when the bulbs were dry-stored for about 12 weeks at 5 °C (Boonekamp *et al.*, 1990; Moe and Wickstrøm, 1973). However, the percentage of bulbs showing bud abortion after planting increased suddenly after 26 weeks of storage, implying that there is a critical storage period regarding the flower potential of the bulb. This is in agreement with the results of Le Nard and De Hertogh (1993), who found bud abortion emerging after 6 months of cold storage for cv. Paul Richter. Although the shoots were not

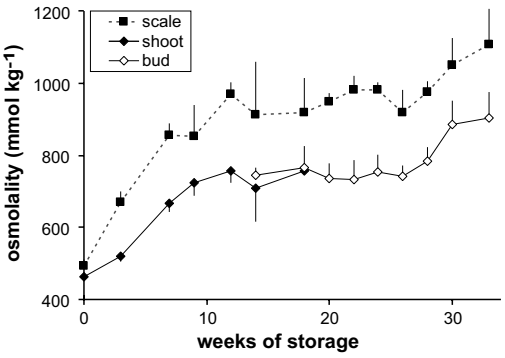


Figure 2.8
Osmolality of the press sap from the second outermost scale and the shoot as a function of storage time. The bud contained the floral tissue above the upper internode of the stem. Error bars represent standard deviations.

able to develop into normal flowers after planting, their growth within the bulb continued at about the same rate over the whole storage period of 34 weeks as appears from the increase in fresh weight and size (Figure 2.6). The T_2-weighted images (Figure 2.3) showed that the morphology/anatomy of the shoots also changed gradually over the whole storage period. A sudden morphological change around 26 weeks of storage, which might have been related to the emergence of bud abortion, was not observed.

Daughter bulbs were visible in T_2-weighted images of bulbs stored for about 14 weeks. They clearly increased in size over the subsequent 20 weeks of storage. Le Nard and De Hertogh (1993) also found that after very long cold storage and subsequent planting the only developmental process taking place was the growth of the daughter bulbs. This occurred after 28 weeks of cold storage in this study. During long cold storage the daughter bulbs obviously grew at the expense of the shoot.

The amount of water in the second outermost scale declined over a period of 34 weeks (data not shown). Since the bulbs were dry-stored, water will have been distributed to the growing shoot and daughter bulbs, both acting as competing sinks for water and metabolites (De Munk and Gijzenberg, 1977; Le Nard and De Hertogh, 1993). Despite of the availability of sufficient external water to sustain growth after planting, aborted flowers had a lower water content (48%) than normal flowers (89%).

The water status of the scale and shoot changed profoundly during storage, as evident from the T_1 and T_2 relaxation times (Figure 2.5). T_2 values in the whole shoot decreased and a minimal level was reached at 22 weeks of storage, which may indicate that the normal development was disturbed from 22 weeks onwards.

Changes in the water status of the stem were also expected because stem elongation, especially in the upper internode, decreased considerably as a result of bud abortion. However, T_1 and T_2 relaxation times in the first internode of the stem were erratic and apparently did not change as a result of the induction of bud abortion. T_2 values in the upper internode did not change during storage and thus do not seem to be indicative for bud abortion either.

The stamens are known to be most sensitive to improper preparation and the first tissue in the bulb to show symptoms of blasting (De Munk, 1973). The T_1 and T_2 values in the stamens decreased continuously (by 38 and 34%, respectively) during the storage period. All bulbs stored for 28 weeks or longer showed bud abortion after planting. This suggests that relaxation times in the stamens below a certain threshold value of T_1 and T_2, which is attained around 26 weeks of storage, could be related to the emergence of bud abortion.

When drawing conclusions from the presented relaxation data, one should keep in mind that the ROI's used for determining the average relaxation value

in the different slices bring a risk of partial volume effects due to the slice thickness used and tissue heterogeneities, especially in the basal plate and the stamens. Nevertheless, the variation between the five bulbs in T_1 and T_2 values in the different ROI's is relatively small. Furthermore it can be concluded that the information provided by these two parameters is different.

The T_1 and T_2 parameters of water in biological tissues depend among others on the water content, interactions with surrounding molecules, diffusion processes and flow. Apart from tissue-intrinsic relaxation processes, the applied temperature, magnetic field strength and pulse sequence variables also affect the measured T_1 and T_2. For example, Van der Toorn *et al.* (2000) found after 12 weeks of storage of tulip bulbs a T_2 value in the scales of 83 ms measured with a multi-echo sequence at 0.47 T and 20 °C. In the present study an average T_2 value of 15 ms was found in the second outermost scale using a spin echo sequence at 4.7 T and 5 °C. Although, a direct comparison of absolute relaxation times should only be done within a similar experimental set-up, trends in relaxation times could be compared. However, most MRI studies on flower bulbs mainly focused on scale tissue of bulbs stored for only a short period of time (Bendel *et al.*, 2001; Iwaya-Inoue *et al.*, 1996; Okubo *et al.*, 1997; Van der Toorn *et al.*, 2000; Yamazaki *et al.*, 1995; Zemah *et al.*, 1999).

The increase of the osmolality in the scale and shoot tissue during storage was probably the result of degradation of starch into sugars and the transport of the sugars from the scales to the shoot (Haaland and Wickstrøm, 1975). Furthermore, the decrease in water content of the shoot and scales will have contributed to an increase in the osmolality. Although Heidema *et al.* (1985) rejected osmolality as a parameter to indicate the fulfillment of the cold requirement, a distinct increase in the osmolality was found during the first 12 weeks of storage (Figure 2.8). In addition, the substantial increase in osmolality in scale and shoot tissue after 26 weeks of storage may reflect aberrations, which appeared in the bulb and will have resulted in bud abortion after long-term storage.

The osmolality showed an overall increase in scale as well as in shoot tissue. Concurrently T_1 and T_2 relaxation times decreased. This coherence might partially be explained in terms of the motional properties of the tissue water. An increase in the osmolality, for instance as a result of an increased sugar content may have decreased the translational and rotational mobility of the water and thus caused a decrease in T_1 and T_2 (Clark *et al.*, 1997; MacFall and Johnson, 1994; Robinson *et al.*, 2000).

Changes in the membrane permeability, assessed via ion leakage measurements might be indicative for the viability of cells (Bonnier *et al.*, 1992). Ion leakage of excised shoot tissue was expected to increase as a result

of a decreasing viability accompanying bud abortion. However, the ion leakage changed during the first 12 weeks of storage, while in the subsequent weeks no changes in ion leakage were found in scale or shoot tissue. This suggests that bud abortion is not associated with changes in tissue viability.

In summary, the development of tulip bulbs during long-term cold storage is accompanied by changes in the water status, indicated by changes in osmolality of tissue sap and T_1 and T_2 relaxation times of the shoot and especially of the stamens. The passing of a threshold value of these parameters might be used as an indication for an increased risk of bud abortion.

Acknowledgements

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Chapter 3

The water status of tulip bulbs during long-term cold storage in relation to bud abortion

M.G. van Kilsdonk, R. van Sluis, C. Kollöffel, K. Nicolay

Abstract

A long-term, low temperature dry storage of tulip bulbs leads to abnormal growth and development of the bud. This disorder, known as bud abortion, manifests itself in tulips with a dehydrated bud or even a fully aborted shoot suggesting a disturbed water status of the bulb. For a better understanding of the processes underlying bud abortion, the water status of tulip bulbs (*Tulipa gesneriana* L., cv. Apeldoorn) was extensively studied, amongst others by magnetic resonance imaging (MRI). T_1 and T_2 relaxation times decreased throughout storage, especially in the stamens, and are discussed in relation to the microscopic structure of the tissue and the composition of tissue sap. The *in vivo* determined water concentration values of the shoot as well as the classically determined water content and osmolality values of the shoot and scale tissue also changed gradually during long-term storage. These changes probably indicate a decreasing availability of water for root and shoot growth directly after planting, implying an increased risk of bud abortion. When the development of the flower bud ceased, growth of the daughter bulbs increased. By the time the shoot had reached the stage of full abortion, water had probably been withdrawn from the bud for growth of the daughter bulbs. Microscopic observations showed degeneration of the stamens after 28 weeks of storage. Furthermore, the water content, water potential and the *in vivo* water concentration in the stamens dropped remarkably, indicating severe changes in the water status of these organs.

Introduction

Water is of paramount importance to plant growth and function. Changes in the water status of a plant can therefore result in a disturbed development and the occurrence of physiological disorders.

For the production of year-round cut flowers, bulbs are exposed to different temperature sequences during storage, which sometimes induce disorders in their development. Many flower bulbs require a period of cold for proper growth and shoot development after planting. In horticultural practice this cold requirement is fulfilled after harvesting. For example 12 weeks at 5 °C is required for the proper flowering of tulip bulbs cv. Apeldoorn (Boonekamp *et al.*, 1990; Heidema *et al.*, 1985; Moe and Wickstrøm, 1973). However, when stored too long at low temperature, bud abortion starts to occur (Chapter 2) (Le Nard and De Hertogh, 1993). Bud abortion in flower bulbs, i.e. ceasing of the development of the shoot within the bulb, is a physiological aberration probably resulting from a disturbed water status. In general, its presence is detected not before planting. Assessment of the internal quality of flower bulbs and detection of aberrations at an early stage of development is, therefore, important in horticultural practice.

Little is known about the cause and course of bud abortion in flower bulbs. Because it can be intentionally induced in tulips, tulip bulbs were used as a model system to assess the course of the disorder. In tulips, bud abortion manifests itself after planting with a short upper internode of the stem and an approximately 1 cm long dehydrated bud with white papery tepals (De Munk and Hoogeterp, 1975; De Munk and Gijzenberg, 1977), and yellow-brownish stamens.

A diversity of techniques can be used to measure various aspects of plant-water relations. However, most measurements require destructive sampling. Magnetic resonance imaging (MRI) offers a non-invasive method for characterizing the water status (Chen *et al.*, 1989; Kim *et al.*, 1999). Longitudinal and transverse relaxation values (T_1 and T_2, respectively) can give information on the mobility of the free water fraction in tissues (Ishida *et al.*, 2000) and have been used to determine the internal quality of fruits and vegetables (Chudek and Hunter, 1997; Clark *et al.*, 1997; Faust *et al.*, 1997).

In a previous study on bud abortion in tulip bulbs, longitudinal measurements of NMR relaxation parameters (T_1 and T_2) and osmolality values of tissue sap indicated changes in the water status that could be related to bud abortion (Chapter 2). The present study focuses on a more extensive characterization of the water status of tulip bulbs (*Tulipa gesneriana* L., cv. Apeldoorn) during long-term dry and cold storage, to obtain a better

understanding of the processes underlying bud abortion. In addition to T_1 and T_2 relaxation times, water concentrations were now also determined by MRI. The biological meaning of the relaxation times as well as the verification of the method for the *in vivo* water concentration determination are described in more detail. The MRI-based parameters were compared with classical water status parameters, including relative water content, solute potential and water potential. Additionally, microscopy was used to assess bulb anatomy. To determine the emergence of bud abortion, bulbs were planted at regular time intervals and a number of bulbs was imaged before planting to directly relate the MRI parameter values to flower quality. Experiments were done in two consecutive years, to study possible year-to-year differences.

Materials and methods

Plant material

Tulip bulbs (*Tulipa gesneriana* L., cv. Apeldoorn), 12-13 cm in circumference, were harvested in July 1999 and July 2000 and stored at 20 °C until two weeks after flower differentiation stage G (Rees, 1973). Subsequently, the bulbs were dry-stored at 5 °C and 70-90% relative humidity (RH) for 36 weeks. The two storage experiments starting in 1999 and 2000 will be referred to as experiment I and II, respectively.

Assessment of flower quality

From 10 weeks until the end of storage, bulbs were planted regularly. At each time point at least 20 bulbs were potted and grown in a ventilated room at 17 °C, 75% RH with a 16 h light period (200 μmol m^{-2} s^{-1}).

Water content

The shoot and three punches of tissue taken from the second outermost scale of 10 bulbs were weighed and dried for 4 weeks at 70 °C. The water content was calculated from the difference between the fresh and dry weight and expressed as a percentage of the fresh weight.

Osmolality

Pieces of the second outermost scale and of the shoot were stored at -20 °C for 2 h, thawed and subsequently squashed during centrifugation (10 min 1000xg) (Chapter 2). After a second centrifugation step (5 min 15800xg) the supernatants were used to determine the osmolality (in mmol kg^{-1}) of the tissue sap with a Wescor Vapory Pressure Osmometer.

Water potential

For the determination of the water potential (Ψ_w), bulb tissue was placed in a small chamber (8 mm diameter) of a dew point microvoltmeter (Wescor HR-33T). After 1 to 2 h of equilibration at 20 °C the Peltier thermocouple was cooled and the dew point measured. The water potential was calculated with a reference curve of different concentrations of KCl. Water potential determinations were done on pieces of the second outermost scale (8 mm diameter, without the epidermis), the upper internode of the stem (slice of 2 mm thickness) and 4 stamens (top 8 mm). Water potential measurements were only performed on tissues obtained during experiment II. After the Ψ_w measurements, the water content of the tissue samples was determined as described above.

Microscopy

After 16 and 28 weeks of storage (experiment II) the stamens were excised from three flower buds. The stamens were fixed with Karnovsky fixative for 24 h, dehydrated with ethanol and embedded in Historesin (LKB, Sweden). Hereafter, the stamens were cut into 2 μm slices with a glass knife using a Reichert OMU3 ultramicrotome. The slices were stained for 7 seconds with a 0.5% Toluidine blue solution in water and examined by light microscopy (Olympus BX50 WI equipped with a Sony DKC 5000 digital camera). The same procedure was carried out for tissue from the second outermost scale at 28 weeks of storage.

NMR imaging

In vivo NMR images were obtained on a 200 MHz NMR instrument (Varian, Palo Alto, CA) interfaced to a 4.7 T, 40 cm horizontal bore magnet. A 85 mm Helmholtz volume coil and a 85 mm birdcage volume coil were used in experiment I and II, respectively. All NMR measurements were done at storage temperature (5 °C) by blowing cold air via a heat exchanger into the coil. After imaging the bulbs were planted as described above.

T_1 and T_2 relaxation times

Images were measured with a multislice spin echo sequence using the following imaging parameters: Field of View (FOV) of 6x6 cm^2; 256x256 matrix (0.2x0.2 mm^2 in plane resolution); 2.5 mm slice thickness; 5 slices, chosen such that the middle slice intersected the bud; 2 transients.

T_2-weighted images were measured with the following parameters: echo times (TE) of 4.8, 5.5, 8, 15 and a last TE of 50 ms (in exp. I) or a last TE of 30 ms (in exp. II); repetition time (TR) of 5.1 s. T_1-weighted images were obtained via the inversion recovery technique using: inversion times (TI) of

0.0007, 0.1, 0.5, 1 and 5 s; TE of 4.8 ms; TR of 5.1 to 25.1 s. T_1 and T_2 maps of the tulip bulbs were obtained by mono-exponential fitting of the T_1- and T_2-weighted images on pixel-by-pixel basis in a program written in Interactive Data Language (IDL) (Research Systems Inc., Boulder USA).

Water concentration

To calculate regional water concentrations, proton density maps of the bulbs were calibrated using an external standard and corrected for position-dependent B_1-field inhomogeneities. Proton density maps of tulip bulbs (only in exp. II) were calculated from the series of T_2-weighted images by extrapolating to zero echo time. Measurements were done in the presence of a capillary (diameter 3 mm) filled with a 40 mg L^{-1} $MnCl_2$ solution. A proton density map of a cylindrical phantom (height 7 cm, 5.5 cm diameter) containing also 40 mg L^{-1} $MnCl_2$ was calculated from series of T_2-weighted images, measured under the same conditions as for the tulip bulbs except for the FOV (9x6 cm^2) and the data matrix (384x256). The proton density maps of the bulbs were divided by the proton density map of the phantom (for B_1-field correction) and divided by the signal intensity of the capillary (for proton density calibration). A pixel intensity of 1 then corresponds to 110 mol protons L^{-1}. When assuming that the MRI signal as measured in the bulb only originates from water protons and that the non-detectable bound water fraction is negligible, this procedure yields the water concentration map (55 mol water L^{-1}, maximal).

To verify the above procedure, the water concentrations determined by MRI were compared with classically determined water concentration values. A quantified volume of homogeneous tissue could not be excised from the bulbs, because the most homogeneous tissue, the scales were too thin and spherical. Therefore, control experiments were carried out on potato tubers. T_2-weighted images of six potatoes (cv. Redstar, commercially obtained) were acquired with the same parameters as described for the tulip bulbs. The TE values were 4.8, 5.5, 8, 15, 30 and 50 ms.

Following the MRI procedure, punches in longitudinal direction were taken out from the central and lateral part of the potato tuber. The volume, fresh and dry weights of the punches were determined and the water concentration in moles per liter of tissue was calculated. These values were found to be linearly correlated with the concentration values calculated via the proton density maps when placing Regions-of-Interest (ROI's) at positions equivalent to the locations of the punches (Figure 3.1). Because the regression coefficient between the *in vivo* and *ex vivo* values was essentially 1.0, it could be concluded that the MRI-method yields reliable estimates of the *in vivo* water concentration.

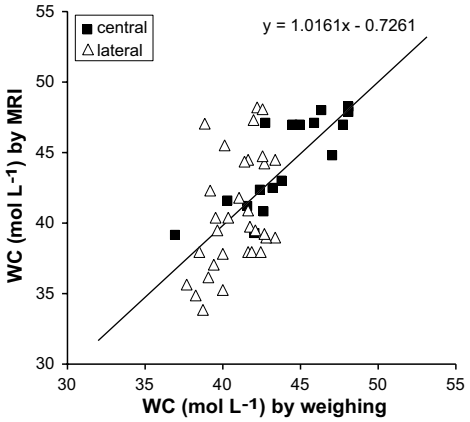

Figure 3.1
Water concentration (WC) values in potato tubers determined by MRI versus the water concentration of the same punches calculated from fresh and dry weights. The equation and line represent the linear regression through the data points of both the central and lateral punches.

The course of the relaxation times T_1 and T_2 as well as the water concentration values of the bulbs in the second outermost scale, the whole shoot, a region in the upper internode of the stem and a region including the stamens and pistil were studied throughout long-term storage (as described in Chapter 2). T_2-weighted images with the shortest TE were used to determine the position of the ROI's. T_1, T_2 and water concentration values of the calculated maps were averaged for each ROI, averaged over the relevant slices through the bulb and averaged over the measured bulbs per time point (n=5).

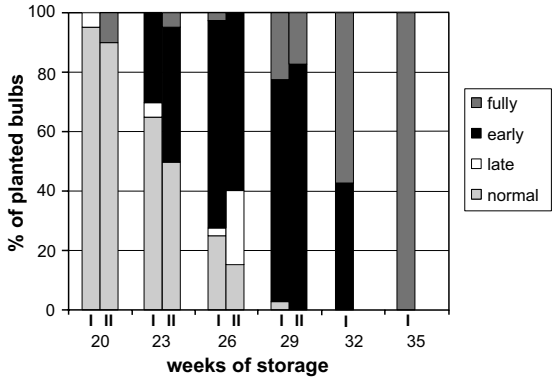

Figure 3.2
Flowering results after dry storage at 5 °C and subsequent planting as a percentage of the total number of planted bulbs (n=20 to 40) for experiment I and II. Flowers on top of an elongated stem with red tepals (5 to 6 cm length), which had opened at anthesis, were designated as normal. Buds with a length of 1.5 to 4 cm with faintly colored tepals, which were not completely dried out were designated as late aborted (late), because bud abortion occurred most probably at a later stage of development than in early aborted buds. Tulips with an approximately 1 cm long bud with unopened, papery white tepals and a short desiccated upper internode of the stem were designated as early aborted (early). Bulbs with a fully aborted shoot (fully) showed hardly any root and stem elongation after planting. Flowering results of the bulbs assessed by MRI are omitted.

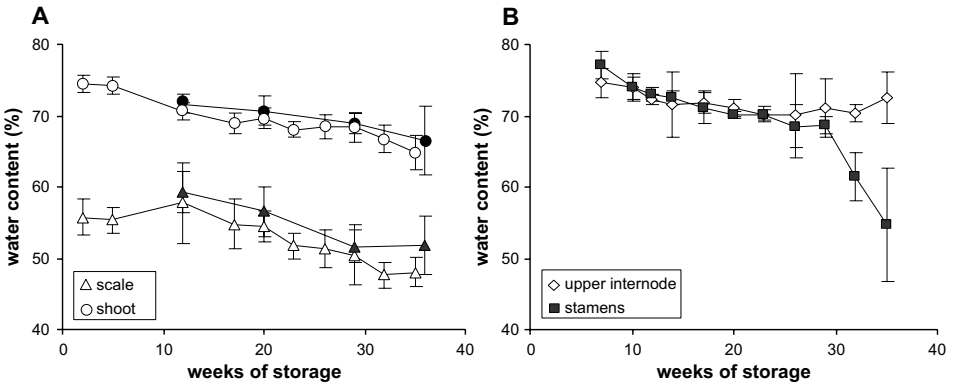

Figure 3.3
Mean water content of (A) the scale and shoot of 10 bulbs during storage (experiment I and II, open and closed symbols) and of (B) the upper internode of the stem and stamens of 5 bulbs during storage (experiment II). The error bars represent the standard deviations.

Results

Flower quality

Nearly all bulbs stored for 10 to 20 weeks at 5 °C developed normal flowers after planting. After 23 weeks of storage and subsequent planting, early bud abortion started to occur and normal flowers were no longer found after 29 weeks of storage in both experiments (Figure 3.2). Bulbs with fully aborted shoots were found at 29 weeks of storage, while all shoots were designated as fully aborted after 35 weeks.

The bulbs screened by MRI and subsequently planted showed slightly worse flower quality. Early bud abortion then occurred in higher percentages (80 and 100% of bud abortion after 27 weeks of storage in experiments I and II, respectively).

During experiment I, some bulbs were also planted on a different location at a lower temperature (15 °C in stead of 17 °C) and less light. Surprisingly, none of the 20 potted bulbs showed bud abortion after 26 weeks of storage. Early bud abortion was now found after 29 weeks of storage in 83% of the planted bulbs, while 12% was still normal and 5% of the bulbs had fully aborted shoots (n=40) (data not shown).

Water content

Over a period of 36 weeks of storage, the bulbs lost on average 45% of their initial fresh weight. This coincided with a similar fresh weight loss in the scales and a 19-fold increase in the fresh weight of the shoot. The water content of shoot and scale tissue decreased gradually throughout storage (Figure 3.3A).

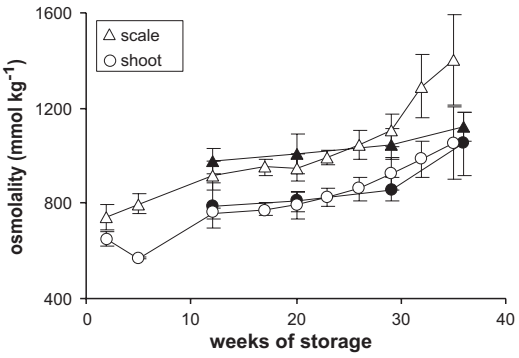

Figure 3.4
Mean osmolality of tissue sap of scale and shoot tissue of 10 bulbs during storage (experiment I and II, open and closed symbols). The error bars represent the standard deviations.

The water content in the upper internode of the stem remained constant during storage while the water content of the stamens decreased slowly until 29 weeks of storage and then decreased dramatically in the next six weeks (Figure 3.3B).

After planting, the water content of early aborted buds was low (48%) compared to that of normal flowers (89%), while late aborted buds had an intermediate water content of 75%.

Osmolality

The osmolality of sap from scale and shoot tissue increased slowly during dry storage of the bulbs (Figure 3.4). After 20 weeks a more pronounced increase in osmolality was found, except for the scale tissue in experiment II.

Water potential

The water potential (Ψ_w) of the scale, the upper internode of the stem and the stamens are depicted as a function of storage time in Figure 3.5. Until 20 weeks of storage Ψ_w hardly changed in all tissues examined. Until 32 weeks, the lowest Ψ_w value was found in the scale. In the upper internode of the stem, hardly any changes in Ψ_w were found. In the stamens, Ψ_w slightly decreased from -1.1 to -1.5 MPa during the first 29 weeks, followed by a sharp decrease to a value of -3.3 MPa at 35 weeks of storage.

Microscopy

Figure 3.6A shows a typical cross-section of a stamen at 16 weeks of storage. Large amounts of starch grains are present in the regularly shaped parenchyma cells. When comparing this cross-section with that of a stamen at 28 weeks of storage (Figure 3.6B), it is obvious that starch grains have disappeared and some parenchyma cells lost their regular shape. At 28 weeks, parenchyma cells surrounding the vascular bundle showed extensive signs of damage. Figure 3.6C shows a cross-section of a scale at 28 weeks of storage, in which a

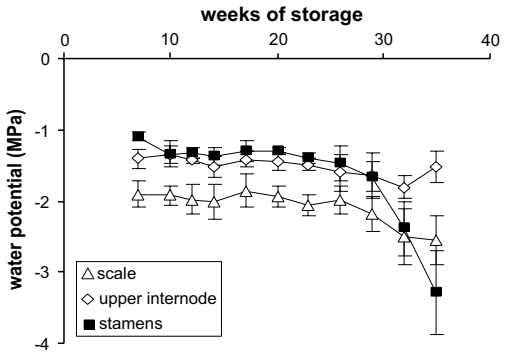

Figure 3.5
Mean water potential of the second outermost scale, upper internode of the stem and stamens of 5 bulbs at different time-points during storage (experiment II). The error bars represent the standard deviations.

compressed layer of empty and degenerated cells between the intact epidermis and the central part of the scale are evident.

NMR-images and relaxation times

Typical examples of parametric images of T_1, T_2 and water concentration of tulip bulbs at 12, 23 and 35 weeks of storage (experiment II) are shown in Figure 3.7. The growth of the shoot during storage can be clearly seen. In both experiments daughter bulbs were first observed after 12 weeks of storage.

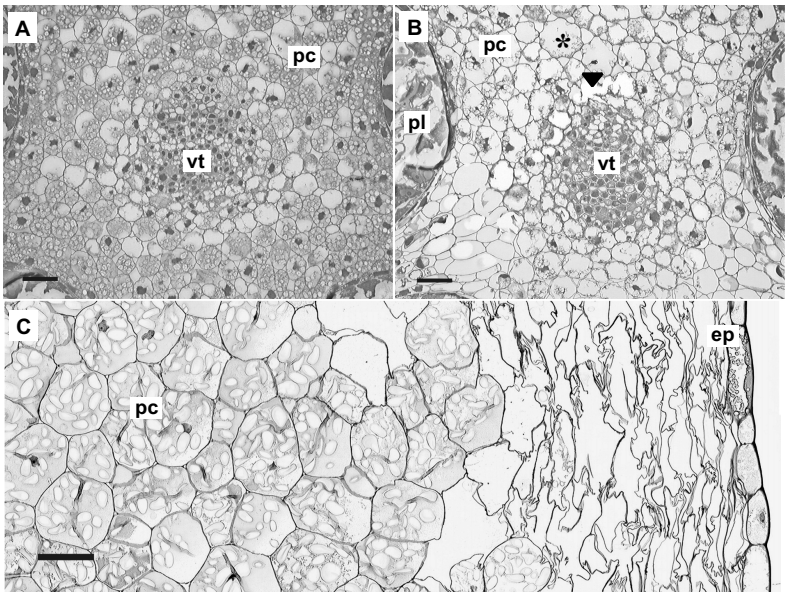

Figure 3.6
Cross-sections of stamens of 16 (A) and 28 (B) weeks stored bulbs and that of the second outermost scale of 28 weeks stored bulbs (C) showing parenchyma cells (pc), vascular tissue (vt), pollen locule (pl) and the epidermis (ep). An example of an irregular shaped parenchyma cell is marked by * and a ruptured cell is marked by ▼. Bars represent 100 µm.

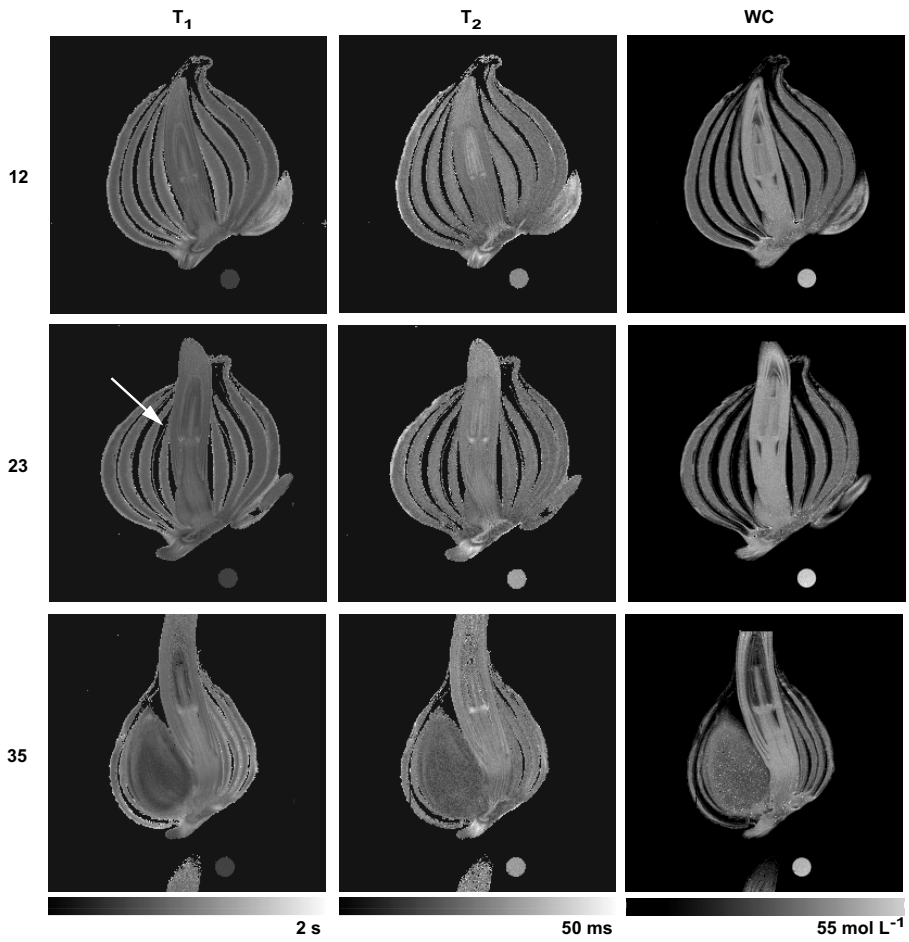

Figure 3.7
Parametric images of T_1, T_2 and water concentration (WC) of tulip bulbs as measured by MRI at three time-points during storage (experiment II): before (12 weeks), around (23 weeks) and after (35 weeks) the emergence of bud abortion. The arrow marks the layer of degenerated cells on the adaxial site of one of the scales. When the shoots became too large, the top was wrapped around to the bottom of the image. Due to inhomogeneity corrections this happened sooner in images of the WC. The T_1 and T_2 relaxation times of the $MnCl_2$ solution in the capillary were 0.26 s and 18 ms, respectively.

Especially the large size of the daughter bulb at 35 weeks of storage is remarkable.

In all images the epidermis of the scales is visible as a rim of high intensity. After 23 weeks of storage a space between the epidermis and the remaining scale tissue appeared at the adaxial site of the scales (Figure 3.7). This space is probably devoid of water because no MR signal was detected. The epidermis and the degenerated tissue layer were omitted from the quantification of the changes of T_1 and T_2 relaxation times and the water concentration in the scale. Average T_1 values of the scale tissue hardly changed during storage (Figure

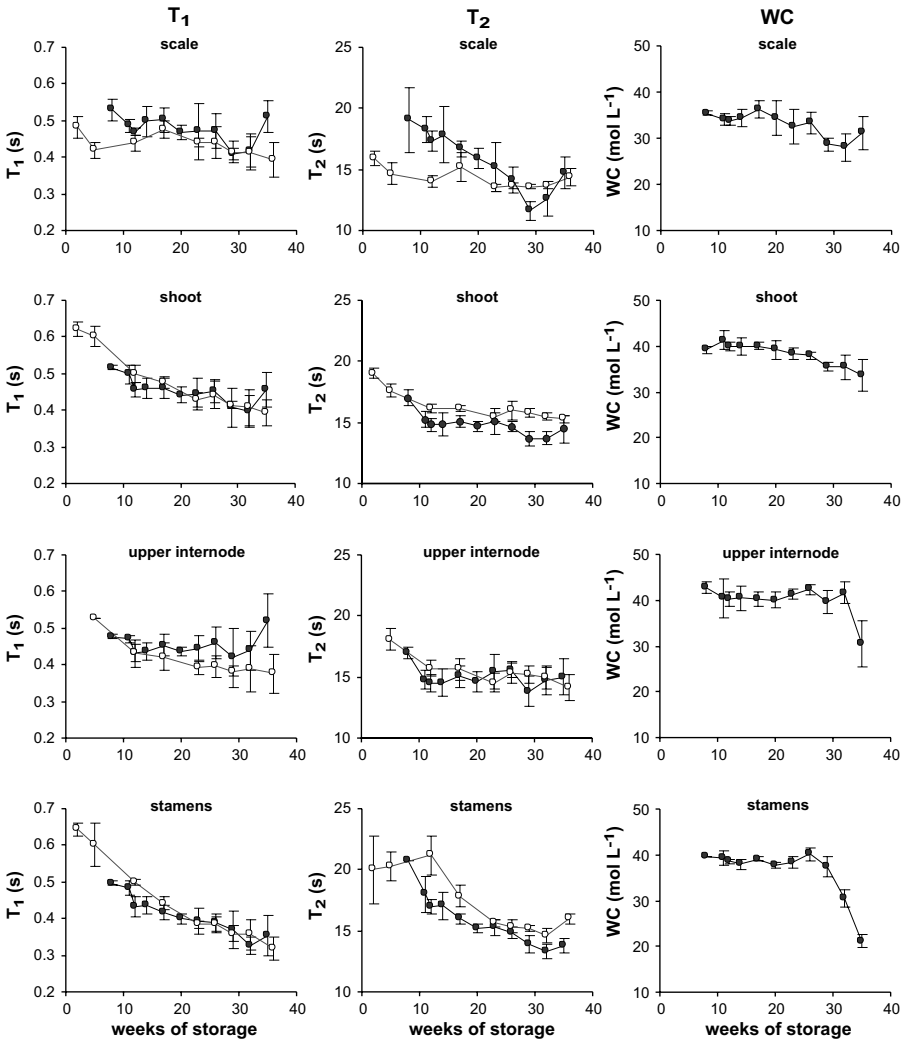

Figure 3.8
Mean values of T_1, T_2 and water concentration (WC) of 5 bulbs within the second outermost scale, the whole shoot and within the upper internode of the stem and stamens plus pistil area as a function of storage time (experiment I and II, open and closed symbols). The error bars represent the standard deviations.

3.8). In the shoot, T_1 values decreased during the first 12 weeks of storage whereupon only a slight decrease was observed, except for the last time point in experiment II, showing a small increase in T_1. A similar increase in T_1 was found in the scales as well as in the upper internode at the end of storage in experiment II. The course of T_1 relaxation times in the upper internode differed somewhat between experiment I and II. In both experiments, the T_1 relaxation times in the stamens plus pistil showed a similar strong decrease.

A remarkable decrease in T_2 values until 29 weeks of storage was also seen in the scale during experiment II (Figure 3.8). In experiment I, however, the T_2 relaxation times of scale tissue remained essentially constant throughout storage. In the shoot as well as in the upper internode the T_2 values showed very little change from 12 weeks of storage in either experiment. A pronounced decrease was found in the T_2 values of the stamens plus pistil in both experiments.

MRI-based water concentrations

The contrast in water concentration (WC) maps (Figure 3.7) is determined by the differences in the number of protons per image-element. Low contrast in the WC maps was found at the beginning of storage suggesting that the WC values of the different tissues inside the bulb were very similar. Average WC values in the different tissues were largely constant during the first 17 weeks of storage (Figure 3.8), whereupon the WC values in the scale and shoot decreased slightly. The WC in the upper internode decreased sharply from 32 weeks and that in the stamens from 29 weeks of storage.

Discussion

Although the origin of bud abortion in tulips might be of metabolic or hormonal nature (De Munk and Gijzenberg, 1977; Moe, 1979) it may be expected that the process of bud abortion is closely related to changes in the water status because dehydration of the flower is the final result. Therefore, various factors contributing to the water status were studied during long-term cold storage of tulip bulbs, which results in bud abortion.

Water status

The whole bulb showed a large weight loss during the cold storage, mainly as a result of water loss by evaporation. The scales, the storage organs of the bulb, supply water and dry matter to the shoot as well as to the daughter bulbs. Water and metabolites were first withdrawn from the adaxial cell layers of the scales as can be concluded from Figure 3.6C and 3.7. The shoot developed continuously during storage (Figure 3.7). The shoot's increase in fresh weight resulted from an increase in dry weight and an increase in the amount of water, yet the water content decreased gradually (Figure 3.3A). Changes in the water content within the bulb coincided with changes in the water concentration (Figure 3.3 and 3.8). Both the water content and the water concentration data indicated that severe dehydration of the stamens (plus pistil) occurred from 29 weeks of storage.

Changes in water potential simultaneously found in the stamens (Figure 3.5) also indicate large changes in the water status. The decrease found in the water potential of the stamens could be a result of the decrease in the water content as it affects both the osmotic potential and the pressure potential. The decreases found in the water content of scale and shoot tissue appear to coincide with the increases in osmolality values, and thus with decreases in osmotic potentials.

The osmolality values (Figure 3.4) indicate a lower (more negative) osmotic potential in the scale tissue than in the shoot. Until 29 weeks of storage, the water potential in the scale was also lower than in the floral tissue (Figure 3.5). This argues against water transport from the scale to the shoot organs via the xylem. Transport of water and solutes to the shoot is probably maintained by the phloem.

To maintain the cell water balance in plant tissues, the availability of the mobile water fraction is important (Slavík, 1974). The mobility of water is decreased by the presence of starch, solutes and cell walls. Mobile water is characterized by relatively high values of T_1 and T_2, while shorter relaxation times are indicative of stronger interactions of water with macromolecules and solutes (Bendel *et al.*, 2001). Also in flower bulbs, T_1 and T_2 relaxation times have been used to study water mobility (Bendel *et al.*, 2001; Robinson *et al.*, 2000; Zemah *et al.*, 1999). Most of these studies addressed the cold requirement of tulip bulbs and encompassed only a short period of storage (approximately 12 weeks) (Iwaya-Inoue *et al.*, 1996; Okubo *et al.*, 1997; Van der Toorn *et al.*, 2000).

The long-term changes in T_1 and T_2 relaxation times found in the shoot and stamens plus pistil (Figure 3.8) suggest a decrease in the mobility of water in the cells. Similar changes were found in a previous study on bud abortion (Chapter 2). During cold storage, starch was degraded (Figure 3.6) into sugars (Haaland and Wickstrøm, 1975), which may have contributed to increased solute concentrations in the shoot (Figure 3.4). High solute concentrations increase the viscosity and the number of interaction sites and thus decrease the relaxation times (Bendel *et al.*, 2001; Callaghan *et al.*, 1994). Furthermore, the composition of the tissue sap may have an important effect on T_1 and T_2 relaxation times, whereas compartmentalization and susceptibility effects also influence T_2 but not T_1 relaxation (Addendum to chapter 3). Changes in these effects, at least in the stamens, are expected because empty, ruptured cells were present in sections of 28 weeks old stamens (Figure 3.6).

Previously, a number of studies have suggested correlations between T_1 and T_2 relaxation parameters and the classical water status parameters (MacFall *et al.*, 1990; Nagarajan *et al.*, 1993; Veres *et al.*, 1991). However, in the present study no relations were found between the relaxation parameters and the water

content or water potential. This is particularly clear in the stamens (Figure 3.5 and 3.8). Nevertheless, a relation was found between the flower quality and T_1 and T_2 values in the stamens. Bulbs, measured by MRI and planted afterwards, showed a lower T_1 and T_2 in the stamens with a reduced flower quality ranging from normal flowers to fully aborted shoots.

Although the observed decreases in T_1 and T_2 relaxation in the shoot and stamens are not fully understood, they seem to be indicative of a gradual change in water status and of reduced flower quality throughout storage time.

Bud abortion

Since weather conditions in springtime may influence the development of tulip bulbs at harvest (De Hertogh and Le Nard, 1993), storage experiments were carried out in two consecutive years. In both experimental years, bud abortion was inducible in the same way and to the same extent. Furthermore, water content, relaxation times and osmolality values (except for the scales) changed similarly throughout the two years. Corresponding results were reported in a previous study on bud abortion (Chapter 2).

Bud abortion emerged after 20 weeks of cold storage and subsequent planting. At longer storage periods, the percentage of aborted flowers and the extent of damage increased (Figure 3.2). The incidence of flower bud abortion is the result of a combined effect of storage period and planting conditions. The latter is indicated by the flowering results after planting at lower temperatures.

During long-term dry storage, the shoot and daughter bulbs develop gradually, while the root primordia hardly grow. After potting, roots and shoot are elongating at a high rate. Water, needed for elongation, is probably first withdrawn from the bulb itself and later on also from the soil. Bud abortion might take place when water is not sufficiently available for shoot growth because the scales are exhausted, the roots are not fully developed or because the sink strength of the daughter bulbs becomes too large. During storage, the mobility of water in the stamens, as determined from the relaxation times as well the water content, water concentration and osmolality values in the scales and shoot decreased continuously. This may be associated with a decreasing accessibility of water within the bulb for growth of the shoot and thus with an increasing risk of bud abortion after planting.

However, when planted at lower temperatures, growth of the roots is enhanced compared to that of the shoot (Le Nard and De Hertogh, 1993), which may enable the bulb to overcome its water deficiency by water uptake from the soil and flower properly.

This seems no longer possible in fully aborted shoots appearing after 29 weeks of storage. These bulbs do not seem to be able to initiate root or shoot

elongation, probably because water can not be made available within the bulb. From that point, all investments will be made to the vegetative reproductive organs, the daughter bulbs. Water and metabolites are probably withdrawn from the shoot to support the growth of the daughter bulbs. This withdrawal of water from the shoot was first detectable in the stamens, in which the water content, water potential and the water concentration dropped dramatically from 29 weeks of storage (Figure 3.3B, 3.5 and 3.8) and degeneration of the parenchyma cells was observed (Figure 3.6).

Acknowledgements

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Addendum to chapter 3

Magnetic resonance relaxation processes in tulip bulbs

Introduction

During long-term cold storage of tulip bulbs, the T_1 and T_2 relaxation times of water within the different tissues change considerably. Unfortunately, the biological meaning of T_1 and T_2 relaxation times in plant tissue, and particularly in flower bulbs, is still poorly understood and more background information on the processes contributing to NMR relaxation is needed.

The T_1 and T_2 relaxation processes of water protons in plant tissue are influenced by multiple factors. Interactions between water protons and surrounding molecules (water itself as well as solutes and macromolecules) affect the motional properties of water protons and alter the rates of relaxation. Thus, changes in the chemical composition of cell sap can cause changes in T_1 and T_2.

Furthermore, plant cells consist of different compartments including the vacuole, cytoplasm and apoplast. The compartments have different sizes and compositions of solutes, and thus different intrinsic relaxation times. Water exchanges between the cellular compartments, leading to a complex averaging of the relaxation times of the individual compartments.

In plant tissues, the extracellular compartments can also be filled with air. Because aqueous solutions and air have different magnetic susceptibilities, a local magnetic field gradient arises at the air-water interface. Diffusion of water molecules through these interfaces causes loss of phase coherence and therefore an increase in T_2 relaxation rate (Köckenberger, 2001). With a used image resolution of 0.2x0.2 mm^2, the NMR signal of individual pixels originates from a large number of cells and a correspondingly large number of cell compartments. The measured T_1 and T_2, although affected by the microscopic structure of the tissue, are thus characteristic of the water status at the macroscopic level.

The relative importance of the different factors contributing to nuclear magnetic relaxation in plant tissue, e.g., chemical composition, subcellular compartmentalization and susceptibility effects, is different for each plant tissue. For example in the parenchyma of apples, compartmentalization and susceptibility effects were found to largely determine T_2 relaxation (Hills and Remigereau, 1997). In the parenchyma of carrots, it was found that the solute composition is the major determinant of the T_2 relaxation time (Hills and Nott, 1999).

For a better understanding of the factors contributing to T_1 and T_2 relaxation in tulip bulbs, *in vivo* T_1 and T_2 data were compared with the relaxation times of tissue saps collected from squashed tissues. The saps were subjected to centrifugation to remove cell debris. In these press sap samples, compart-mentalization and magnetic susceptibility effects are absent and will no longer contribute to relaxation.

Furthermore, the T_1 and T_2 of sucrose solutions were measured, since sugars are known to be a major component of the solutes in tissue sap of tulip bulbs. During cold storage, sap from the shoot has an osmolality between 600 and 1200 mmol kg^{-1}, which can be accounted for 40-50% to sugars (glucose, fructose and mainly sucrose), 20-30% to ions (mainly K^+) and 10-15% to amino-acids (unpublished results).

Materials and methods

In vivo T_1 and T_2 relaxation data of the scale and shoot were obtained as previously described (see NMR-imaging in main text).

Press sap samples of the scale and shoot from 2 bulbs were collected after 2, 12 and 35 weeks of cold storage during experiment I. During experiment II, press sap samples of the scale and shoot of 3 bulbs were obtained after 12, 20, 29 and 35 weeks of cold storage (see Osmolality in main text).

T_1 and T_2 relaxation measurements of the press saps were carried out on a 200 MHz, 4.7 T NMR system (Varian, Palo Alto, CA). For T_2 measurements a Hahn spin-echo pulse sequence with a series of TE's was used, in which the first echo time of 4.8 ms was followed by 12 steps of 5 ms using a TR of 3 s. For T_1 measurements a series of inversion times of 0.0007, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 s was used with a TE of 4.8 ms and TR of 5.1 s. Four transients were averaged. Measurements were done at 5 °C. T_1 and T_2 were calculated by mono-exponential fitting procedures.

T_1 and T_2 relaxation of sucrose solutions of 298, 590, 897 and 1275 mmol kg^{-1} were measured in the same way as described for the press sap samples. For the 298 mmol kg^{-1} sucrose solution, a TR of 10 s was used as well as a series of TE's starting at 4.8 ms and using 20 increments of 5 ms for T_2 measurements. For the other sucrose solutions a TR of 7 s was used.

Results and discussion

In Figure 3.9, the T_1 and T_2 values of the press sap samples of scale and shoot tissue are shown together with the relaxation data of tulip bulbs obtained *in vivo*. It is evident that the *in vivo* T_1 and the T_1 in press sap samples are

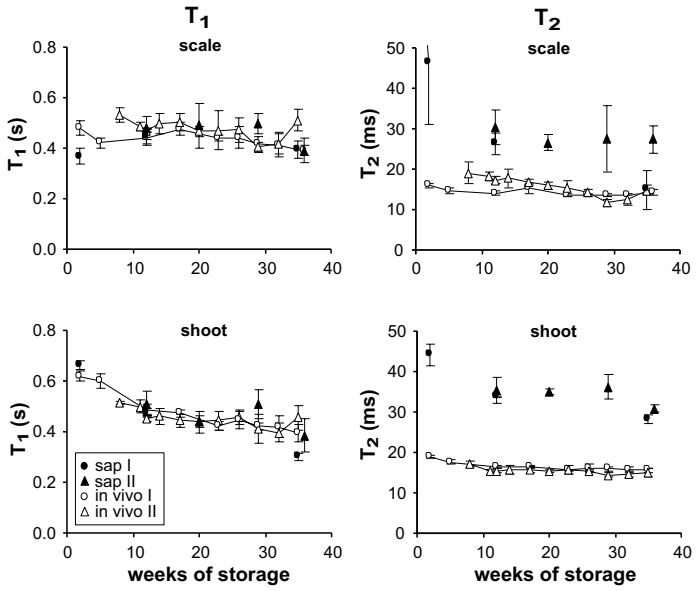

Figure 3.9
Average T_1 and T_2 relaxation times of the second outermost scale and shoot obtained *in vivo* (open symbols) and in sap samples (closed symbols) during storage of tulip bulbs. Data are presented from experiment I and II. The error bars represent the standard deviations.

Table 3.1
T_1 and T_2 values of different sucrose solutions measured at 5 °C

sucrose solution (mmol kg^{-1})	sucrose solution (mM)	T_1 (s)	T_2 (ms)
298	272	1.34	186
590	497	1.09	138
897	701	0.90	78
1275	914	0.71	61

essentially equal. This implies that compartmentalization and susceptibility effects have no significant effect on T_1 relaxation processes *in vivo*. The T_2 values in the press sap samples of the scale and shoot were considerably higher than T_2 values measured *in vivo* (Figure 3.9). This indicates that apart from the chemical composition, the combined effect of compartmentalization, susceptibility gradients and interactions with macromolecules, removed by centrifugation of the press saps, must have contributed significantly to T_2 relaxation *in vivo*. The contribution of these factors was larger in the shoots than in the scale tissue.

Sugars represent almost half of the sap constituents. However, at *in vivo* concentrations (300-600 mmol kg^{-1}), sucrose solutions (Table 3.1) had a much higher T_1 and T_2 than the tissue saps (Figure 3.9). Even at the osmolality levels of the tissue saps (600-1200 mmol kg^{-1}), the sucrose solutions had a higher T_1 and T_2 (Table 3.1). Apparently, the sugar component of the sap samples was not the major contributor to T_1 and T_2 relaxation. The presence of ions, especially paramagnetic ions (Fe, Cu), might be important.

T_1 and T_2 were almost linearly dependent on the sucrose concentration. In sap samples, T_1 and T_2 did not change linearly with increasing osmolality (data not shown). This would suggest that the relative concentrations of the components contributing to T_1 and T_2 relaxation changed with changing osmolality. In summary, T_1 relaxation in tulip bulb tissue is mainly determined by the chemical composition of the tissue sap whereas T_2 relaxation is also affected by subcellular compartmentalization and/or susceptibility gradients.

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Chapter 4

The effects of storage at high temperatures on the quality of tulip bulbs

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Submitted

Abstract

In horticultural practice temperature regimes are used to control flowering and bulb propagation. This may enhance the risk of physiological disorders like bud abortion in tulip bulbs, i.e. the failure of a bulb to produce a marketable flower. The detection of these disorders at an early stage of development is thus an important issue. Bud abortion in tulip bulbs (*Tulipa gesneriana* L. cv. Apeldoorn) was induced by dry-storage of bulbs at high temperatures (30 and 35 °C). A subsequent period of low (5 °C) temperatures was applied to fulfill the cold requirement. During the high-temperature pretreatment, growth of the shoot was arrested, while during the subsequently applied cold storage, the shoots enlarged rapidly. As a result of the storage at high temperatures, a wide variety of flower aberrations emerged after planting. With increasing storage temperature and duration, flower quality shifted from normal flowers, to flowers with shriveled tepals and eventually to stems without a flower. The water content of the aberrant flowers after planting was normal (89%). The ion-leakage from the shoots of stored bulbs increased with the duration of the high-temperature pretreatment and seems therefore indicative for a decreased flower quality. With the use of magnetic resonance imaging, aberrations in the flowers could be visualized non-invasively with T_2-weighted images after the cold requirement was fulfilled. The effects of the storage temperatures on the T_1 and T_2 relaxation times were ambiguous. Bud abortion induced by high storage temperatures probably involves processes, different from in those observed previously after long-term, dry-storage at 5 °C. Thus the term bud abortion is too general, as different aberration patterns should be distinguished.

Introduction

In horticultural practice flower bulbs are exposed to a wide variety of temperature regimes to control flowering and bulb propagation. Exposure of bulbs to inappropriate temperatures during storage or planting enhances the risk of physiological disorders. To minimize financial losses to flower bulb growers, there is great need to detect these aberrations at an early stage of development.

Bud abortion is such a disorder. In tulip bulbs it can be induced by long-term cold storage, storage at high temperatures and by exposure to ethylene (De Munk and Hoogeterp, 1975; Le Nard and De Hertogh, 1993). Long-term cold storage was found to result in changes in the water status of stored bulbs and in dry necrotic flowers after planting (Chapter 2). In the present study, the effects of storage at high temperatures are studied to assess parameters that could be indicative for the internal flower quality at an early stage of development. Therefore, the development of bulbs exposed to high temperatures (30 and 35 °C) was compared to that of bulbs, dry-stored at moderate (20 °C) or low (5 °C) temperatures. Because bulbs need a minimal period of cold for root and stem elongation after planting (Gilford and Rees, 1973), bulbs stored at moderate and high temperatures were subsequently given a period of 12 weeks of cold to fulfill this requirement.

High-temperature treatments induce the growth of the daughter bulbs (Le Nard and De Hertogh, 1993). Application of a high-temperature treatment to abort the flower and to increase daughter bulb production is actually used in practice to propagate the planting stock (Koster, 1980; Rees, 1967).

NMR-imaging was used to study the development of the shoot and daughter bulbs during storage. This technique enables *in vivo* cross sectioning and a clear distinction between different types of tissue based on water content and water properties (Chapter 3). Maps of longitudinal and transverse relaxation times (T_1 and T_2, respectively) were measured to obtain quantitative information on the water status of the different tissues. Since these relaxation processes are temperature sensitive and because different storage and measuring-temperatures were used, the temperature dependence of the relaxation times was studied additionally. Flower quality after planting was assessed to determine the damage caused by the storage protocol. Various parameters including the water content, osmolality, ion-leakage and relaxation times were assessed to establish their potential predictive value for the quality of the flowers after planting.

Material and methods

Plant material

Tulip bulbs (*Tulipa gesneriana* L., cv. Apeldoorn), 12-13 cm in circumference, were harvested in July 1998 (year I) and 1999 (year II) and stored at 20 °C until two weeks after flower differentiation stage G (Rees, 1973).

The different succeeding storage procedures used in the present study are depicted in Figure 4.1. In year I, the bulbs were dry-stored either at 5, 20 or 30 °C. The storage at 5 °C up to 32 weeks is described in Chapter 2. The storage at 20 °C lasted 10 weeks. After 4 and 8 weeks of storage at 20 °C the bulbs were subsequently stored at 5 °C for 11 weeks and planted. The storage at 30 °C lasted 4 and 8 weeks and was followed by storage at 5 °C for 11 weeks.

In year II the bulbs were dry-stored at 5 °C or 35 °C. Storage at 5 °C up to 36 weeks is described in Chapter 3. Storage at 35 °C lasted 3, 6, 9 or 13 weeks and was followed by 12 weeks of storage at 5 °C.

The storage treatments during the first 13 weeks at low, moderate and high temperatures will be indicated as '5'-, '20'- and '30/35'-pretreatments. Hereafter, the cold requirement of the bulbs was fulfilled with 12 weeks of cold (5 °C) storage, which will be indicated as the '5+5'-, '20+5'- or '30/35+5'-treatments.

Following moderate- or high-temperature storage and the 12 weeks of cold storage, bulbs (n=20) were potted and grown in a ventilated room at 17 °C, 75% RH and 16 hr light period (200 μmol m^{-2} s^{-1}) to assess flower quality.

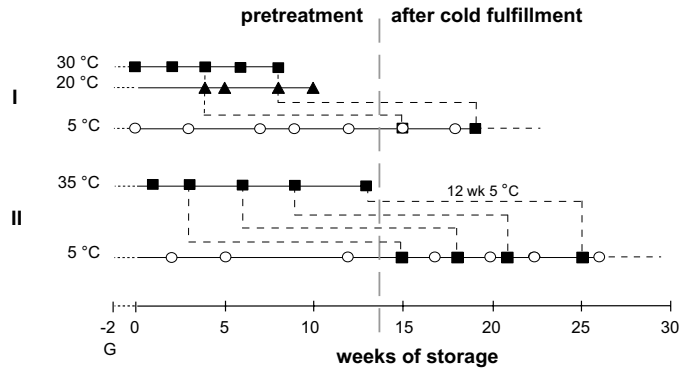

Figure 4.1

Scheme of the temperature sequences and sampling points used in year I and II. The sampling points of the bulbs pretreated with high (30/35 °C), moderate (20 °C) and low (5 °C) temperatures are indicated with a ■, ▲ and ○, respectively. At different time points, the high and moderate temperature pretreated bulbs were transferred to low temperatures for 12 weeks (dashed lines) to fulfill the cold requirement.

The water content of flowers after planting (at anthesis) was determined, additionally.

Water content

After weighing and 4 weeks of drying at 70 °C, the water content of the shoot and that of three punches of the second outermost scale of 10 bulbs was calculated as the difference between the fresh and dry weight and expressed on a fresh weight basis.

Osmolality

Shoot and second outermost scale tissue were stored for 2 h at -20 °C and squashed during a centrifugation step (10 min 1000xg) after thawing (described in Chapter 2). After another centrifugation step (5 min 15800xg) the osmolality of 10 µL of the supernatant was determined with a thermocouple psychrometer (Wescor Vapory Pressure Osmometer). Until 8 weeks of storage (year I), the press saps of the shoots were pooled before the first centrifugation step.

Ion-leakage

A disc (13 mm) from the second outermost scale was punched out, weighed and incubated in 50 mL of distilled water per gram tissue. After 24 h of incubation at 20 °C, the ion-leakage, measured as the conductivity of the incubation medium, was determined with a conductivity meter (Radiometer Copenhagen). The same procedure was followed for the whole shoot and the flower bud (the part of the shoot above the first internode).

NMR-imaging

T_2-weighted images were obtained on a 4.7 T (200 MHz) 40 cm horizontal bore magnet (Varian, Palo Alto, CA) and measured with the following parameter settings for a Hahn spin-echo sequence: Field of View 6x6 cm^2; 256x256 matrix, 2.5 mm slice thickness, 5 slices (middle slice through the bud), 2 transients, echo time (TE) of 5 ms (in year I) and 4.8 ms (in year II) and a repetition time (TR) of 5 s.

To calculate the T_2 maps the same procedure was carried out with a series of TE's of 5, 7, 10, 15, 20 ms in year I and 4.8, 5.5, 8, 15, 30 ms in year II. For the T_1 maps different inversion times (TI) were used: 0.001, 0.2, 0.5, 1, 5 s (TE of 5 ms, TR 5 to 10 s) in year I and in year II 0.0007, 0.1, 0.5, 1, 5 s (TE of 4.8 ms, TR 5.1 to 25.1 s).

T_1 and T_2 maps were obtained by mono-exponential fitting of the T_1 and T_2-weighted images on a pixel-by-pixel basis using a program written in Interactive Data Language (IDL).

In year I, the same bulbs were used at every time point to study longitudinal developments whereas in year II different bulbs were used each time, which were planted afterwards. At every time point 4 to 5 bulbs were measured.

Regions-of-Interest (ROI's) were positioned in the T_2-weighted images (shortest TE) and include an area in the second outermost scale, in the basal plate, in the first internode of the stem, a region including the stamens plus pistil and the whole shoot (Figure 2.1). The mean relaxation times in the different ROI's were averaged over the (relevant) slices through the bulb and over the different bulbs measured.

Bulbs were imaged at the same temperature at which they were stored. Temperatures during imaging were controlled by blowing air via a heat exchanger into the cylinder in which the bulbs were fixed. To determine the effect of the measuring-temperature on the relaxation times of different tissues, 'ice-tulips' were acclimated and imaged at 5 and 35 °C. Ice-tulips were used because their flower development and quality remains approximately the same over a few months because they are stored at freezing temperatures. To obtain ice-tulips, bulbs (cv. Apeldoorn) (at stage G) were dry stored at approximately 20 °C for 12 weeks, then for 6 weeks at 5 °C and finally for at least 6 weeks at -2 °C, packed in soil. The T_1 and T_2 measurements were performed as described above.

Results

Flower development

Based on the characteristics after planting, the flowers were classified into the following eight categories of increasing abnormality (Figure 4.2):

Category 1: Normal flower with red tepals (approximately 6.5 cm length) on top of an elongated stem (approximately 30 cm length). Flowers with minor abnormalities like aborted stamens, somewhat pale and/or irregularly shaped tepals were also classified into this category.

Category 2: Flower with red to pale red tepals (approximately 6 cm length), often irregularly shaped, narrow and not fully unfolded on top of an elongated stem (approximately 30 cm length).

Category 3: Flower with red to yellow to green tepals (approximately 3 cm length), highly irregularly shaped, narrow, not fully unfolded and wrinkled, on top of an elongated stem (approximately 25 cm length). Occasionally, the pistil and stamens were absent and sometimes a large pistil (approximately 2.5 cm) was found.

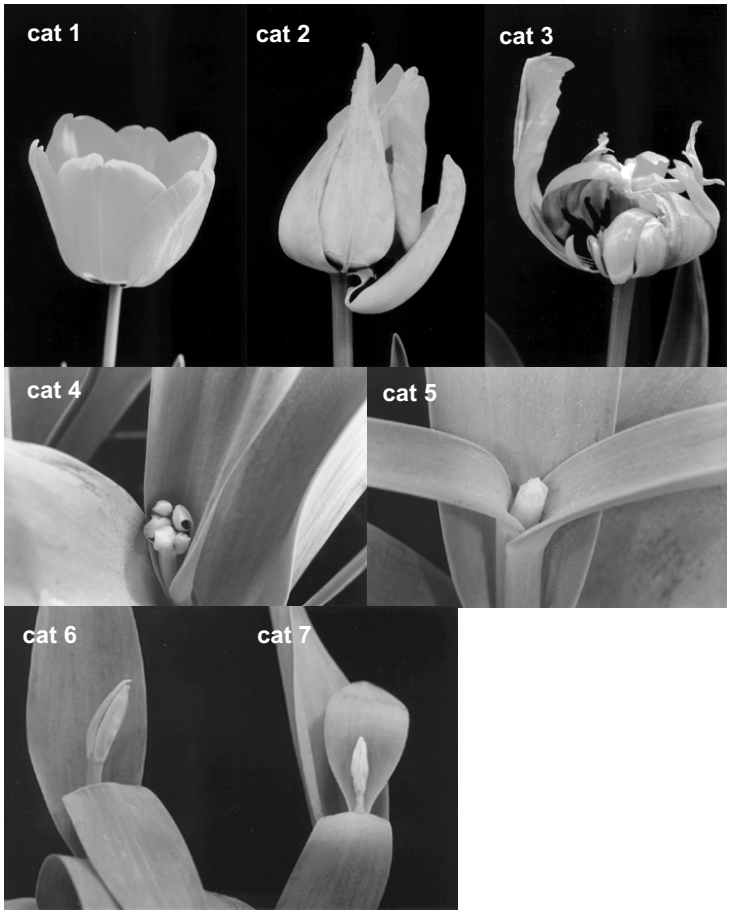

Figure 4.2
Photographs of flowers, illustrative of the different categories of flowers found after planting of bulbs stored at moderate and high temperatures and subsequently low temperatures. Descriptions are given in Results.

Category 4: 'Flower' (approximately 1 cm height) with mostly green crumpled tepals on top of a usually not fully elongated stem (approximately 10 cm height). The leaves had developed normally.

Category 5: Short stem (approximately 7 cm) with normal green leaves, without an elongated upper internode and without a flower.

Category 6: Flower with rather dehydrated tepals (approximately 3 cm length) usually pale green or with red and purple spots on top of an upper internode with variable length. Total stem length was approximately 16 cm. The leaves had developed normally.

Category 7: Flower with dehydrated, white tepals (approximately 1 to 2 cm length) and a dehydrated pistil and dry, yellow-brownish stamens.

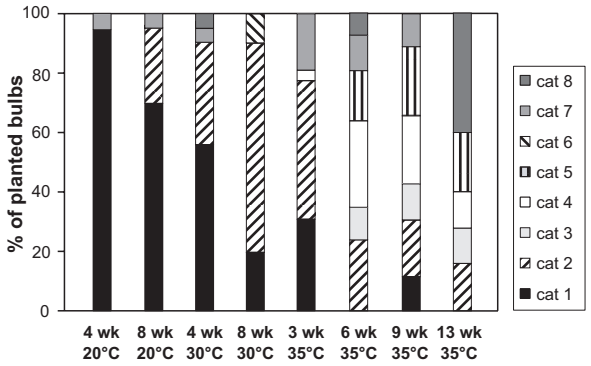

Figure 4.3
Flowering results after pre-treatment at 20, 30 and 35 °C, followed by 12 weeks of storage at 5 °C and subsequently planting, presented as percentage of the planted bulbs (n=20). Categories are described in Results and illustrated in Figure 4.2.

The upper internode of the stem was dehydrated and not elongated (approximately 1.5 cm length) while the leaves were normal. Total stem length was approximately 8 cm.

Category 8: Shoot did not emergence after planting.

The effects of the moderate- and high-temperature pretreatment on the flower quality after planting are shown in Figure 4.3. After 4 weeks of storage at 20 °C and subsequent cold, the flowers were essentially normal (category 1). With increasing storage temperature and duration, the flower characteristics shifted gradually from category 1 to 5. Eventually, after 13 weeks of storage at 35 °C, 40% of the shoots did not emerge at all after planting (category 8).

The water contents of the different flowers after planting are presented in Table 4.1. Flowers of category 1 to 4 had similar water contents whereas the water content of flowers of category 7 was much lower.

Table 4.1
Water content (mean ± standard deviation) of a number of flowers (n) of planted bulbs. Flowers are classified into the different categories as shown in Figure 4.2. The data of the normal flowers (category 1) concern mostly long-term cold stored bulbs. (n.d. not determined)

	water content (%)	n
category 1	88.7 ± 1.1	29
category 2	88.3 ± 1.1	6
category 3	89.6 ± 1.5	3
category 4	86.3 ± 1.5	7
category 5	n.d.	
category 6	n.d	
category 7	58.5 ± 5.3	4
category 8	n.d.	

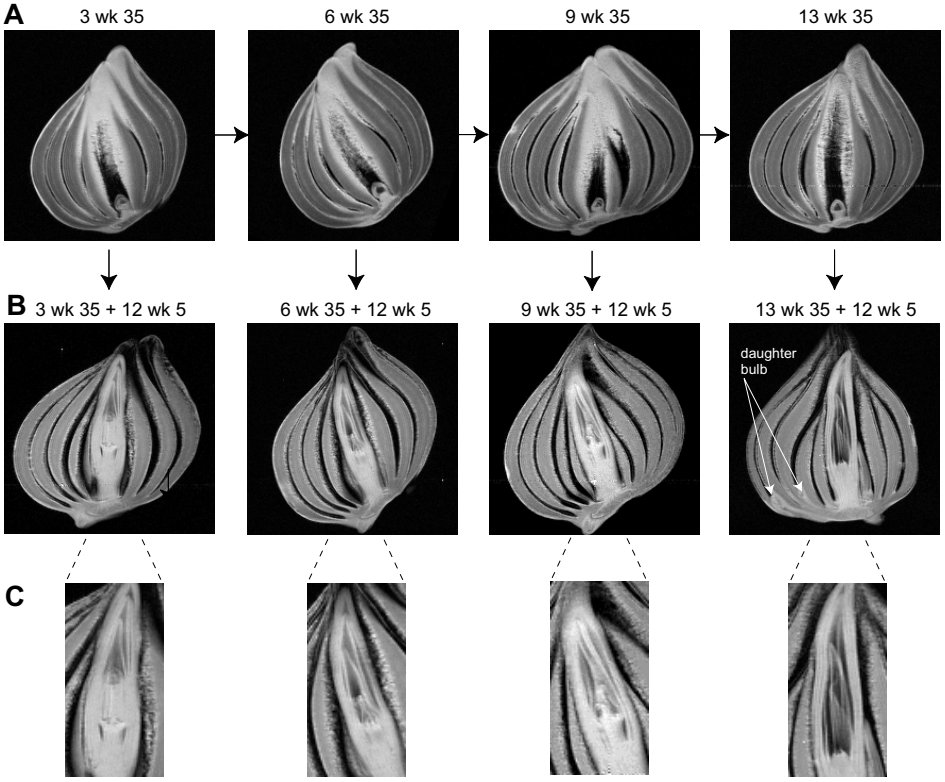

Figure 4.4
Typical examples of T_2-weighted images (TE = 4.8 ms, TR 5.1 s) of different bulbs during 13 weeks of storage at 35 °C (A) and after subsequently 12 weeks of storage at 5 °C (B) during year II. The buds of the bulbs in B are enlarged in C.

Root growth after planting appeared to be normal for all categories (not determined for category 8). Bulbs of all categories except for category 8 developed 1 to 5 leaves per bulb originating from the daughter bulbs (not determined for category 6). The number of 'daughter' leaves increased slightly (from 2 to 3 on average) with increasing severity of the floral aberrations.

Below, the development of the bulbs during the '5'-, '20'- and '30/35'-pretreatments will be described separately from the development after the subsequent cold fulfillment ('5+5'- and '30/35+5'-treatments) (Figure 4.1).

Growth and development

During pretreatment

The T_2-weighted images of '35'-pretreated bulbs show that the shoots remained small during storage (Figure 4.4). Changes in shoot morphology as

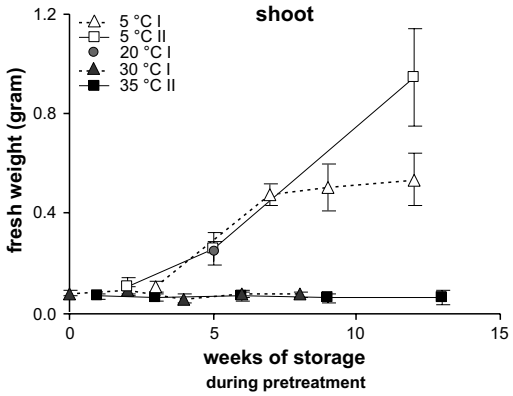

Figure 4.5
Mean fresh weights of the shoots (n=10) during the period of pretreatment in year I and II. The data of the cold treatments were taken from Chapter 2 and 3. The error bars represent the standard deviations.

a result of increasing storage time were not observed. The fresh weights of the shoots of '30/35'-pretreated bulbs remained unchanged during the pretreatment, while those of the '20'- and '5'-pretreated bulbs increased considerably (Figure 4.5).

During and after cold fulfillment

During the subsequent cold storage, the fresh weights of the shoots of high-temperature pretreated bulbs had increased considerably. The fresh weight of '30/35+5'-treated shoots was now comparable to that of '5'-pretreated bulbs at 12 weeks of storage (data not shown).

The NMR images also show that growth of the shoot had occurred during the cold fulfillment (Figure 4.4). Independent of the pretreatment temperatures, the stem and the leaves had developed similarly during the cold fulfillment. However, the flower buds showed large differences. Whereas '20+5'-treated bulbs developed into normal flowers, aberrations were observed in '30/35+5'-treated bulbs. Some buds had regular large tepals and stamens (e.g. Figure 4.4B/C, 3 wk 35 + 12 wk 5) while other ones had (very) small tepals and stamens (e.g. Figure 4.4B/C, 6 wk 35 + 12 wk 5). Occasionally, all structures resembling floral parts were absent (e.g. Figure 4.4B/C, 13 wk 35 + 12 wk 5). On the images of the '30/35+5'-treated bulbs, no daughter bulbs in the axils of the scales were observed, except for one of the '35+5'-treated bulbs stored for 25 weeks (Figure 4.4B/C, 13 wk 35 + 12 wk 5). After 9 weeks of 35 °C and subsequent cold, bulbs were also examined *ex vivo*. Longitudinal sections of the shoot showed a brown necrotic area below the normally yellow-colored flower bud (Figure 4.6).

The morphological characteristics of the bud as observed on the NMR-images of dry-stored bulbs seem to be related to the symptoms found after planting. Bulbs with floral parts, which were visible on the images, had flowers

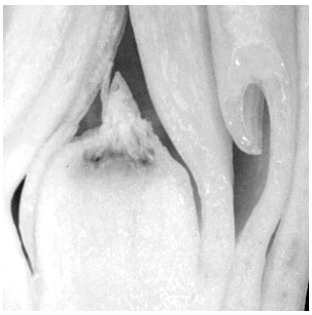

Figure 4.6
Longitudinal section of a shoot from a bulb stored for 9 weeks at 35 °C and subsequently 12 weeks at 5 °C. The tissue was yellow-colored except in the area below the flower bud, which was brown.

assigned to category 2 and 3 after planting. Bulbs with an apparently empty space above the upper internode, resulted after planting in flower stalks of category 4 and 5.

Water content

During pretreatment

The water contents in the scale and shoot of '30/35'-pretreated bulbs were approximately 2 to 3% lower than those in the '5'-pretreated bulbs of the same year (data not shown). However, for similar conditions, there was a difference in water content of more than 10% between the two years. The highest water contents were found in the '5'-pretreated bulbs in year I (approximately 70% in the scale and 85% in the shoot).

After cold fulfillment

In the scale, the differences in the water content between the two experimental years were still present after the cold fulfillment. Furthermore, the water content in the '30/35+5'-treated bulbs was still almost similar to that in the '5+5'-treated bulbs within the same experimental year.
In the shoot of the '30/35+5'-treated bulbs, the water content was approximately 5% higher than that in the shoots of '5+5'-treated bulbs (data not shown).

Osmolality

During pretreatment

The osmolality in the scale (Figure 4.7A) and shoot (Figure 4.7B) increased during the first 12 weeks of storage at every temperature. This increase was higher in the scales of '5'-pretreated bulbs than in moderate- and high-temperature pretreated bulbs (Figure 4.7A). In the shoot the same pattern was found, except for the shoots of '35'-pretreated bulbs, which had a slightly higher osmolality than those of '5'-pretreated bulbs (Figure 4.7B).

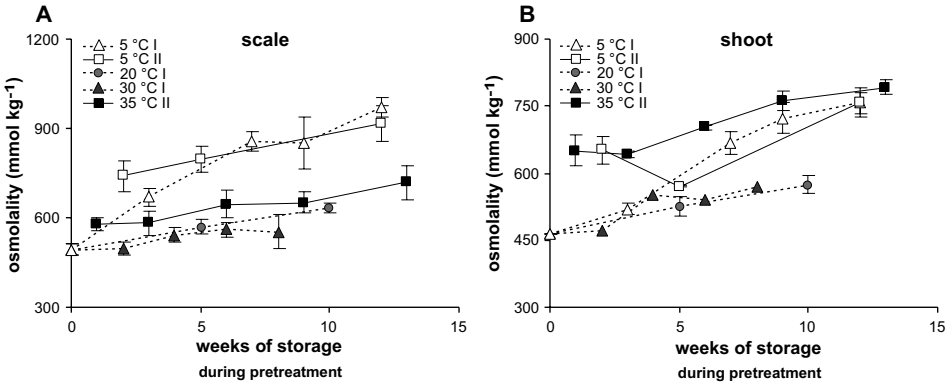

Figure 4.7
Mean osmolality values of the scales (A) and shoots (B) (n=10) during the pretreatment in year I and II. The data of the cold treatments were taken from Chapter 2 and 3. The error bars represent the standard deviations.

After cold fulfillment

After the cold fulfillment, the osmolality values of the scale and shoot increased gradually with increasing storage duration. Differences were not observed among the different pretreatments.

Ion-leakage

During pretreatment

During storage at moderate and high temperatures the ion-leakage from the scale increased slightly (approximately 20 μS cm^{-1} over 12 weeks), while it increased clearly during cold storage (approximately 100 μS cm^{-1} over 12 weeks) (data not shown). In contrast, the ion-leakage from the shoot increased considerably during storage at high temperatures, whereas it decreased somewhat at moderate and low temperatures (Figure 4.8A).

After cold fulfillment

The ion-leakage from the scales after the '30+5'-treatment was similar to that from '5+5'-treated bulbs of the same year (approximately 130 μS cm^{-1}). The ion-leakage from the scales after the '35+5'-treatment was higher than the values obtained from '5+5'-treated bulbs of the same year (180 and 107 μS cm^{-1}, respectively at 25 weeks of storage) (data not shown). After the cold fulfillment, the ion-leakage from the flower buds of '30/35+5'-treated bulbs was still higher than that from the '5+5'-treated bulbs but the ion-leakage had decreased to a large extent (Figure 4.8B). The ion-leakage from the bud tended to increase with the duration of the high-temperature pretreatment, but the variation was large.

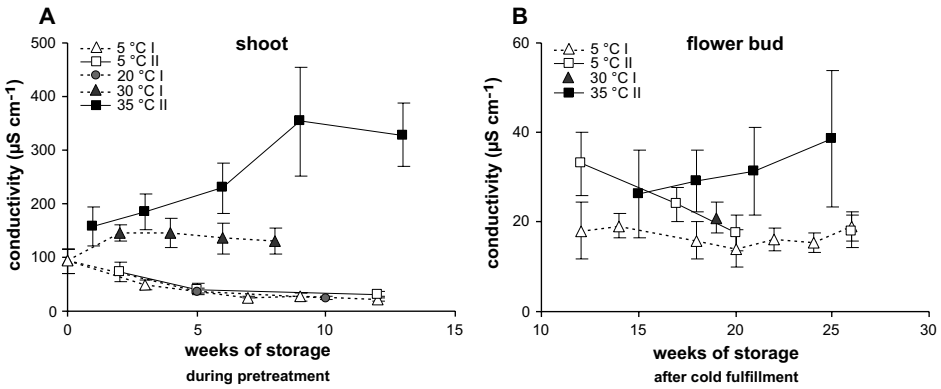

Figure 4.8
Mean conductivity values of the incubation media of the shoots and flower buds (n=10) during the pretreatment (A) and after the cold fulfillment (B). The data of the cold treatments of year I were taken from Chapter 2. The error bars represent the standard deviations.

NMR-relaxation parameters

During pretreatment

During the pretreatment, the T_1 and T_2 relaxation times of the different tissues in the bulb hardly changed. Therefore, only the relaxation times obtained at the end of the high-temperature treatment will be discussed and compared to the values of the '5'-pretreated bulbs (Table 4.2).

In year I, a difference in T_1 in the scales of 0.16 s was found between bulbs stored at low (5 °C) and high (30 °C) temperatures (Table 4.2). This difference amounted to 0.25 s in year II. In ice-tulips a larger difference in T_1 (0.40 s) was found in the scales of at 5 and 35 °C imaged bulbs. This difference is caused purely by the difference in measuring-temperature. Thus, based on the effect of temperature alone, the T_1 in the scale of '30/35'-pretreated bulbs was expected to be higher. Storage effects probably caused a decrease of T_1 in the scales. This was also found in the basal plate. The difference in T_1 of the shoot between '5'- and '30/35'-pretreated bulbs was almost zero (0.01 s) and 0.29 s in year I and II, respectively. In the shoots of ice-tulips, the difference in T_1 was 0.39 s as a result of the different measuring-temperatures. This implies that in the shoot, especially in year I, a T_1 lowering effect was induced by the high-temperature storage. The same effect was found in the stem and stamens of year I.

During year I, the T_2 in the scales of high-temperature stored bulbs was lower than that of cold-treated bulbs (5.1 ms difference), whereas in year II almost no difference was found between the T_2 in the scales of '5'- and '35'-treated bulbs. In the ice-tulips no significant effect on T_2 was found in the

Table 4.2

T_1 and T_2 relaxation times (mean ± standard deviation) in different tissues of bulbs, stored and imaged at low (5 °C) or high (30 and 35 °C) temperatures. In year I, 9 weeks of 5 °C storage was compared to 8 weeks of 30 °C storage. In year II, 12 weeks of 5 °C storage was compared to 13 weeks of 35 °C storage. To determine the effects of the measuring temperature, ice-tulips were measured at 5 and 35 °C.

	pretreatment and measuring temperature				measuring temperature	
	year I		year II		ice-tulips	
	5 °C*	30 °C	5 °C*	35 °C	5 °C	35 °C
T_1 (s)						
scale	0.43 ± 0.01	0.59 ± 0.02	0.44 ± 0.02	0.69 ± 0.02	0.55 ± 0.03	0.95 ± 0.11
basal plate	0.31 ± 0.02	0.60 ± 0.13	0.50 ± 0.01	0.72 ± 0.08	0.56 ± 0.05	1.02 ± 0.09
shoot	0.42 ± 0.02	0.43 ± 0.03	0.50 ± 0.02	0.79 ± 0.10	0.58 ± 0.05	0.97 ± 0.12
stem	0.37 ± 0.03	0.43 ± 0.03	0.50 ± 0.04	0.98 ± 0.03	0.64 ± 0.14	1.11 ± 0.2
stamens	0.41 ± 0.01	0.37 ± 0.03	0.50 ± 0.01	0.84 ± 0.10	0.52 ± 0.02	0.86 ± 0.11
T_2 (ms)						
scale	15.0 ± 1.0	9.9 ± 0.1	14.0 ± 0.5	15.0 ± 0.2	17.7 ± 2.5	20.1 ± 2.4
basal plate	10.8 ± 0.2	10.0 ± 0.4	14.8 ± 0.5	17.0 ± 0.7	13.9 ± 1.0	12.8 ± 0.8
shoot	12.8 ± 0.3	10.9 ± 0.1	16.1 ± 0.4	18.8 ± 0.4	18.3 ± 1.2	16.1 ± 1.0
stem	12.0 ± 0.4	10.8 ± 0.4	15.4 ± 1.0	19.1 ± 0.8	22.0 ± 2.3	18.2 ± 2.3
stamens	14.9 ± 0.3	12.8 ± 0.5	21.2 ± 1.6	17.9 ± 1.2	19.7 ± 0.5	16.7 ± 1.6

***The data from the cold treatments of year I and II were taken from Chapter 2 and 3, respectively.**

scale as a result of the different measuring-temperatures. This suggests that in year I the lower T_2 in the scale can be attributed to high-temperature storage effects. In the shoots of year I, the different pretreatment temperatures did not result in significant differences in T_2, while in year II a higher T_2 value was found at 35 °C storage (2.7 ms difference). In ice-tulips, the T_2 value in the shoot determined at 35 °C was not significantly different from that at 5 °C. In most tissues T_2 was not significantly altered by the measuring-temperature. This implies that the higher T_2 value as found in the '35'-treated shoots of year II was induced by the pretreatment. The basal plate and stem showed the same effects of storage on T_2 as found in the shoot in both years. In the stamens, no pretreatment effects on T_2 were found. In summary, these results imply that in year I, T_1 was decreased due to high storage temperatures, while in year II the storage effects on T_1 were small. In year II, the T_2 was increased in most tissues due to storage at high temperatures, while in year I no such effects were observed.

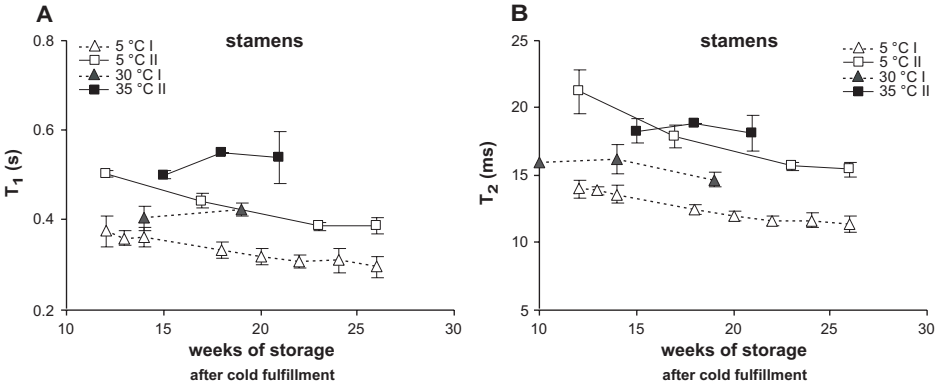

Figure 4.9
Mean T_1 (A) and T_2 (B) values of the stamens (n=4 or 5) after the cold fulfillment. All measurements were done at 5 °C. The data of the cold treatments were taken from Chapter 2 and 3. The error bars represent the standard deviations.

After cold fulfillment

After the cold treatment all bulbs were imaged at 5 °C. In year I, T_1 and T_2 relaxation times in all tissues were lower compared to year II (approximately 0.1 s and 5 ms difference in T_1 and T_2, respectively). The relaxation times of the '30/35+5'- and '5+5'-treated bulbs were similar within the same experimental year, except for the stem and stamens. The T_1 in the stem of '35+5'-treated bulbs was approximately 0.05 s lower than the T_1 in the stem of the '5+5'-treated bulbs (data not shown). In the stamens the '30/35+5'-treatment resulted in higher T_1 and somewhat higher T_2 values than the '5+5'-treatment (Figure 4.9A and B). The difference in T_1 values between the bulbs from different pretreatment protocols increased with increasing duration of the pretreatments: '5+5'-treatment resulted in a decrease, while '30/35+5'-treatment resulted in small increase in T_1.

Discussion

Long-term cold storage of tulip bulbs as well as storage at high temperatures are known to induce bud abortion (Chapter 2) (Le Nard and De Hertogh, 1993; Rees, 1973) and thus to decrease the flower quality of the planting stock. Assessment of the internal quality of flower bulbs during induction of the disorders may lead to the identification of (generally applicable) quality parameters. In this study bud abortion was induced by storage at high temperatures.

Planting data showed that storage of tulip bulbs cv. Apeldoorn at high temperatures induced major disorders in flower development, which increased in severity upon prolongation of the high-temperature treatments. The most prominent morphological aberrations were the shriveling, and finally the disappearance of the tepals. Although damage due to high temperatures, i.e. 'heating in transit' has been described before (Rees, 1967; Rees, 1973), the presented phenomena were not reported previously. Regardless of the extreme aberrations found after planting, the aberrant flowers (category 2 to 4) had similar water contents as normal flowers (89%) (Table 4.1). In contrast, bud abortion induced by long-term cold storage manifested itself with dry necrotic white tepals and dehydrated stamens and pistil (Chapter 2 and 3). These aborted buds had a reduced water content of 48% (Chapter 2). 'Late' aborted buds had a water content of 75% (Chapter 3). Apparently, the characteristics of bud abortion induced by high-temperature treatments and by long-term cold storage differ strongly. Flowers classified into category 6 and 7 are an exception. These flowers do resemble 'late' aborted and 'early' aborted buds after long-term cold storage (Chapter 3) in terms of morphology and water content (Table 4.1). However, such flowers were only found in small numbers after every pretreatment in the present study.

Bud abortion induced by long-term cold storage was shown to be accompanied with changes in the water status during storage, as indicated by changes in the water content, osmotic potential and T_1 and T_2 relaxation times (Chapter 2). These parameters were also assessed for their predictive value for flower quality after storage at high temperatures.

Storage at high temperatures arrested the growth of the shoot (Figure 4.5), while after the subsequent cold fulfillment the growth was fully recovered. The water content of the scale and shoot was not affected by the high temperature pretreatment. Differences in the osmolality were found between the two storage temperatures. The osmolality in the scales of bulbs stored at low temperatures was higher than in bulbs stored at moderate and high temperatures (Figure 4.7), probably because the conversion of starch into sugars is enhanced at low temperatures (Moe and Wickstrøm, 1979). As a possible consequence, the osmolality in the scales became higher than in the shoot during cold storage (Figure 4.7). The osmolality in the scales of the high-temperature pretreated bulbs also increased and became higher than in the shoot during the subsequently applied cold-treatment. So the effects of the high temperature pretreatment were nullified at the end of the cold fulfillment with regard to the osmolality. The osmolality values thus reflect the growth of the shoot during the high plus low temperature sequence but were not related to the aberrations induced.

The decreasing water content and increasing osmolality (and thus an increasing viscosity) during long-term cold storage were assumed to play a role in the relaxation rates, resulting in decreasing T_1 and T_2 values within the shoot (Chapter 2). However, the use of different measuring-temperatures complicated the interpretation of the relaxation times in this study. By measuring the relaxation times in ice-tulips at different temperatures it could be concluded that the measuring-temperature affected T_1 relaxation to a large extent while T_2 relaxation was hardly altered. The effects of the pretreatment were ambiguous. High storage temperatures caused in year I a decrease of T_1, while in year II, T_2 was mostly affected and showed an increase. After the cold fulfillment, the stamens, when present, could indicate the effects of the high temperature pretreatment.

Thus, during storage, the discussed water status parameters (water content, osmolality, T_1 and T_2) are not indicative of flower aberrations induced by storage at high temperatures. However, the ion-leakage from the shoot increased with increasing temperature and duration of the pretreatment (Figure 4.8A and B). The large variation of the data reflects the large variation in aberrations found after planting. Bonnier *et al.* (1992) also found an increased ion-leakage from tulip bulbs due to storage at high temperatures and related this to a decreased viability of the tissue.

T_2-weighted images indicated, except for an arrested growth, no changes in the morphology during storage at high temperatures. The image resolution must be increased in order to visualize disorders in the small floral parts. After the applied cold treatment, however, irreversible damage to the flower was clearly visible. The extent of abnormalities in the floral parts visible in the images of stored bulbs was related to the severity of damage assessed after planting. During long-term cold storage, no changes in the shoot morphology and ion-leakage were found (Chapter 2).

As a result of the high temperature treatment, symptoms of necrotic processes were found below the flower bud (Figure 4.6), which may be related to the arrested development of the floral parts. This area could not be distinguished in the MR-images probably because of the small size. Endogenous ethylene may play a role in the processes induced by storage at high temperature (Kanneworff and Van der Plas, 1994; Rees, 1973).

In contrast to the long-term cold storage experiments (Chapter 3), a large variation in most of the assessed parameters was found between the two years. The use of a reference value of healthy, normal bulbs when assessing the quality of bulbs should be considered.

High temperatures are used to propagate the planting stock, because they reduce the dominance of the central daughter bulb and thus induce growth of a large number of daughter bulbs compared to low storage temperatures

(Koster, 1980; Le Nard and De Hertogh, 1993). During long-term cold storage, the rapid growth of a single dominant daughter bulb was clearly visible on MR images of all stored bulbs (Chapter 2 and 3). After the high-temperature pretreatments, only one bulb with growing daughter bulbs was found (Figure 4.4B 13 wk 35 + 12 wk 5). After planting, however, the number of daughter bulbs producing a leaf was similar for high-temperature pretreated bulbs and for bulbs after long-term cold storage (unpublished results). Daughter bulbs produce a leaf only after reaching a certain size. Thus, damage to the flower bud in general results in an increased number of 'large' daughter bulbs after planting, though the distribution of the sizes of the daughter bulbs after each treatment may be different.

In this study it was also found that the osmolality and ion-leakage values in the scales of the cold-treated bulbs were different from the values in the scales of bulbs stored at moderate temperatures stored, while the storage at high and moderate temperatures resulted in comparable values. Regarding the shoots, the high temperature treated bulbs reacted differently from the bulbs stored at low and moderate temperatures. This implies that the cold storage mainly affected the scales, while heat-stress largely affected the shoot.

Summarizing, in contrast to long-term cold storage, storage of tulip bulbs at high temperatures resulted in an altered morphological development and an enhanced ion-leakage, while the water status was not changed. Thus, it can be concluded that not only the symptoms of bud abortion after storage at high temperatures and after long-term cold storage are different, also different physiological processes are probably involved.

The term bud abortion or blasting, i.e. the failure of a bulb to produce a marketable flower after the floral organs have been formed (De Hertogh and Le Nard, 1993) covers the disorders as found after storage at high temperatures and long-term cold storage, but it is a very general term. Since the underlying processes appear to be different, more specific definitions could and should be used to indicate the different physiological disorders.

Acknowledgments

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Chapter 5

Freezing injury in stored Oriental hybrid lily bulbs

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Submitted

Abstract

Oriental hybrid lily bulbs are stored at subzero temperatures for long periods for the year-round production of flowers. After harvest and a precooling period, the bulbs are frozen in moist peat. The start of the freezing period and the freezing temperature should be chosen properly to minimize the risk of freezing injury. However, the optimal timing and temperature conditions are variable between cultivars and years and freezing injury can not be entirely prevented. To assess the effects of freezing injury in lily bulbs, the storage of Oriental hybrid *Lilium* bulbs cv. Star Gazer at subzero temperatures was delayed (6 weeks later than control bulbs) and a lower temperature than usual (-4 °C instead of -1 °C) was applied. Only prolonged storage at -4 °C resulted in freezing damage, manifesting with aberrant leaves and eventually aborted shoots. The decreased water content and water potential show that freezing damage involves dehydration of the damaged tissue. The decreased longitudinal relaxation time (T_1) in the stem and shoot apex of freezing damaged bulbs, determined non-invasively by magnetic resonance imaging, also pointed towards desiccation of the tissue. Changes in the water status of the damaged tissue were not reflected in changes in the transverse relaxation (T_2). The increasing ion-leakage from the shoot during storage at too low temperatures, probably indicating changes in membrane permeability, coincided with the increased damage found after planting the bulbs. Changes in the ion-leakage of the scales and in the osmolality of the shoot as found are probably more indicative of acclimation to the applied storage temperatures.

Introduction

For the year-round production of flowers, lily bulbs are stored at subzero temperatures for long periods to minimize the growth of the shoot. After harvest in late autumn, Oriental hybrid lily bulbs are generally subjected to a low temperature pretreatment (0 to 4 °C) for eight to ten weeks, whereupon in early spring the temperatures are lowered to -0.5 to -1.5 °C for months (Wilkins and Dole, 1997). To prevent freezing injury, freezing must be carried out at the proper physiological state of the shoot and at the proper temperatures. However, these requirements are different for each cultivar and harvest (Gude *et al.*, 2000a; Kok *et al.*, 2001).

During the low temperature pretreatment, freezing tolerance is increased by increased sugar levels in the bulb and has its optimum somewhere in early spring (Kok *et al.*, 2001). The increased sugar content possibly decreases the freezing point of the tissues (Gusta *et al.*, 1996; Jones *et al.*, 1999; Sauter *et al.*, 1996). When the maximum sugar levels are attained and freezing tolerance is maximal, storage at subzero temperatures can be started. The bulbs are frozen in moist peat to minimize damage due to desiccation.

When storage at subzero temperatures is started before the maximal freezing tolerance is reached, freezing injury may occur. Besides, when the pretreatment at low temperatures is prolonged beyond the point of maximal sugar content, growth and development of the shoot continue and freezing tolerance decreases (Kok *et al.*, 2001). Furthermore, when the applied freezing temperature decreases below a certain threshold temperature, freezing injury emerges after planting.

Freezing injury of the shoot of lily bulbs results after planting at first in abnormal growth of the lowest leaves of the stalk and in the worst case by a plant without proper stem elongation and without flowers (Gude *et al.*, 2000a). Especially, when a leaf or flower meristem is damaged during the freezing period, large effects can be seen after growth of the plant in the greenhouse.

During storage at the usual freezing temperatures (-0.5 to -1.5 °C), the bulbs do not actually freeze in general (Miller and Langhans, 1990). Freezing injury is mostly the result of the presence of ice-crystals in/or surrounding a cell. The formation of intracellular ice crystals can destroy the intracellular compartmentalization and consequently damage the tissue whereas extracellular ice-crystals can cause desiccation to the intracellular compartments (Burke *et al.*, 1976; Steponkus, 1984). Growth of ice-crystals in the soil and tissue might withdraw water from the plant cells, finally resulting in desiccated tissues (Burke *et al.*, 1976). Changes in the water content might thus be indicative of freezing damage.

When intracellular ice-crystals are formed, ruptures in the plasmalemma and tonoplast result in loss of cellullar compartmentalization, causing a decreased viability of the tissue. Damage to the membranes can lead to leakage of cell saps and components. Leakage of ions into an incubation medium can easily be detected by measuring the conductivity of the incubation medium (Bonnier *et al.*, 1997; Forney and Peterson, 1990).

It has been suggested that ruptures in cell membranes can also be determined non-invasively by magnetic resonance imaging (MRI) (Duce *et al.*, 1992). Longitudinal and transverse relaxation times (T_1 and T_2, respectively) determined by MRI are influenced by the mobility of water molecules (Bendel *et al.*, 2001; Ishida *et al.*, 2000). As a result of dehydration and ruptures in membranes the mobility of water is altered which can be reflected in altered relaxation times (Hills and Nott, 1999; Kaku *et al.*, 1985). Apart from that, increasing sugar concentrations also decrease the mobility of water molecules (Chapter 3) (Hills and Nott, 1999).

The purpose of this study was to assess the processes involved in freezing injury in stored lily bulbs. To obtain control flowers, lily bulbs were stored at -1 °C starting in February. To induce freezing injury lily bulbs were frozen late (6 weeks later) or frozen at lower temperatures (-4 °C) compared to the control procedure. The damage induced by the temperature sequence was verified by determining the plant and flower quality after planting the bulbs in a phytotron with constant light intensity, temperature and humidity. These planting conditions may also influence the flower quality after planting (Erwin and Heins, 1990; Lee and Roh, 2001) and variation in these factors was thus prevented. The parameters assessed to evaluate freezing damage were the water content, osmotic value, ion-leakage, water potential and NMR relaxation times. Most parameters were assessed in two consecutive years, to incorporate year-to-year effects.

Materials and methods

Plant material

Oriental hybrid *Lilium* bulbs cv. Star Gazer, 14-16 cm in circumference were harvested at the end of November 1998 (year I) and 1999 (year II) and stored in moist peat at 2 °C (year I) or 0 °C (year II) until early February.

Before freezing the bulbs were disinfected for 15 minutes. Bulbs were left to drain for at least half an hour. Hereafter the bulbs were packed per 10 in plastic bags filled with moist peat. The storage experiments were started at 3 and 5 February 1999 and 2000, respectively. The temperature sequences of the

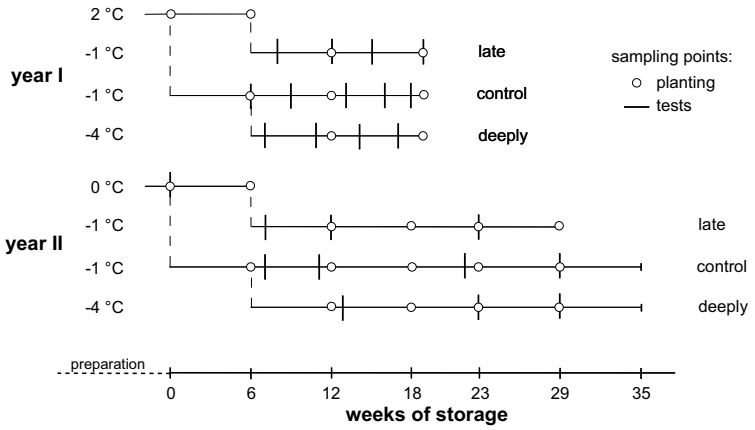

Figure 5.1
Scheme of temperature sequences of control, late and deeply frozen bulbs and the sampling points used in year I and II. After harvest, bulbs were stored at preparation temperatures of 2 and 0 °C in year I and II, respectively. Storage experiments started at 3 and 5 February 1999 and 2000. Bulbs were transferred to -1 °C or remained another 6 weeks at 2 (year I) or 0 °C (year II) (late frozen). At 6 weeks of storage a number of control bulbs (-1 °C) was transferred to -4 °C (deeply frozen).

different experiments are schematically depicted in Figure 5.1. Before the measurements, the bulbs were left to thaw at room temperature during a night. Subsequently, the mother scales, enclosing the new bulb, were removed.

Planting

Bulbs (including the mother scales) were planted at a depth of approximately 5 cm in 20 cm deep pots. The bulbs were grown in a ventilated room at 17 °C, 75% RH and 16 hr light period (200 μmol m^{-2} s^{-1}). The number of days before flowering of the first bud (DTF), the number of buds and the stalk height at flowering were determined. Aberrations in the flower and leaf development were also noted.

Water content

The water content of the shoot and that of the combined three outermost scales of the new bulb (n=10) were calculated as the difference between the fresh and dry weight, expressed on a fresh weight basis, after weighing and 4 weeks of drying at 70 °C.

Osmolality of tissue sap

The shoot was stored for 2 h at -20 °C and squashed during a centrifugation step (10 min 1000xg) after thawing (described in Chapter 2). After another

centrifugation step (5 min 15800xg) the osmolality of 10 μL of the supernatant was determined with a thermocouple psychrometer (Wescor Vapory Pressure Osmometer).

Ion-leakage

Discs (13 mm) from the three outermost scales of the new bulb were punched out, combined, weighed and incubated in 50 mL of distilled water per gram tissue. After 24 h of incubation at 20 °C, the ion-leakage, measured as the conductivity of the incubation medium, was determined with a conductivity meter (Radiometer Copenhagen). The same procedure was carried out for the shoot (without the basal plate).

Water potential

For the determination of the water potential (Ψ_w), pieces of the basal plate and the shoot apex were placed in a small chamber (8 mm diameter) of a dew point microvoltmeter (Wescor HR-33T). After 3 h of equilibration at 20 °C the Peltier thermocouple was cooled and the dew point measured. The water potential was calculated with a reference curve of different concentrations of KCl. The water content of both the basal plate and the shoot apex were determined following the water potential measurements.

NMR-imaging

In vivo NMR images were obtained at 29 weeks of storage (year II) on a 200 MHz NMR instrument (Varian, Palo Alto, CA) interfaced to a 4.7 T, 40 cm horizontal bore magnet. A 85 mm Helmholtz volume coil was used. NMR measurements were done at 20 °C.

Images were obtained with a spin echo sequence using the following imaging parameters: Field of View (FOV) of 6x6 cm^2; 256x256 matrix (0.2x0.2 mm^2 in plane resolution); 2.5 mm slice thickness; 5 slices, 2 transients.

T_2-weighted images were measured using: echo times (TE) of 4.8, 5.5, 8, 15, 30 ms; repetition time (TR) of 5.1 s. T_1-weighted images were obtained via the inversion recovery technique using: inversion times (TI) of 0.0007, 0.1, 0.5, 1 and 5 s; TE of 4.8 ms; TR of 5.1 to 25.1 s. T_1 and T_2 maps of the lily bulbs were obtained by mono-exponential fitting of the T_1- and T_2-weighted images on a pixel-by-pixel basis using a program written in Interactive Data Language (IDL).

The T_2-weighted images with the shortest TE and intersecting the bud were used to determine the position of the Regions-of-Interest (ROI's). T_1 and T_2 values were averaged for each ROI and averaged over the measured bulbs per treatment (n=3).

Table 5.1
Number of flowers and plant aberrations originating from 20 planted bulbs after different temperature sequences and subsequent planting during year I and II.

year	treatment	weeks of storage	sampling point	flowers*	flowers per stalk*	aborted buds	aberrant flowers	stalks with aberrant leaves	aborted shoot	aberrant** stalks
I	control	0	start	68	3.6					
		6	(6 wk -1)	63	3.2	1				
		12	(12 wk -1)	56	2.9	3	3		1	6
		19	(19 wk -1)	48	2.5	2	4		1	6
	late	6	(6 wk +2)	64	3.2	2				
		12	(6 wk +2) + (6 wk -1)	61	3.2		1			1
		19	(6 wk +2) + (13 wk -1)	58	3.1	3	6		1	10
	deeply	12	(6 wk -1) + (6 wk -4)	62	3.1	7	1			7
		19	(6 wk -1) + (13 wk -4)	68	3.4	8	5	17		20
II	control	0	start	68	3.6					
		6	(6 wk -1)	70	3.5					
		12	(12 wk -1)	73	3.7	1	3			4
		18	(18 wk -1)	70	3.5					
		22	(22 wk -1)	64	3.2					
		29	(29 wk -1)	62	3.1					
	late	6	(6 wk +0)	75	3.8					
		12	(6 wk +0) + (6 wk -1)	76	3.8	1	2			2
		18	(6 wk +0) + (12 wk -1)	61	3.1	1				
		22	(6 wk +0) + (16 wk -1)	63	3.3		2		1	2
		29	(6 wk +0) + (23 wk -1)	63	3.2		4	1		4
	deeply	12	(6 wk -1) + (6 wk -4)	70	3.5	3	1			4
		18	(6 wk -1) + (12 wk -4)	77	3.9	1				
		22	(6 wk -1) + (16 wk -4)	68	3.4	2	5	11		15
		29	(6 wk -1) + (23 wk -4)	41	3.2	5	4	6	7	14

*** aborted buds are not included ** stalks with one or more aberrations**

Results

Planting results

Plant quality is determinative for the effectiveness of the temperature sequences (Figure 5.1) to induce freezing injury in Oriental hybrid lily bulbs. The length of the (non-aborted) stalks did not change in relation to the storage duration or temperature. It varied between 45 and 57 cm in year I and between 43 and 51 cm in year II. The number of days to flowering (DTF) of the first bud at the start of the storage experiments (week 0) was 97 and 100 days in year I and II, respectively. The DTF of the control and late frozen bulbs decreased with the duration of the storage and reached a minimum of 86 and 88 days after 19 and 29 weeks of storage in year I and II, respectively. The DTF of bulbs stored at -4 °C decreased with 3 to 7 days in the course of storage. The average number of flowers per stalk from control bulbs decreased from 3.6 to 2.5 and 3.1 in year I and II, respectively, as a result of increasing storage duration (Table 5.1). There was no relation between the freezing temperature and the average number of buds after planting.

Plants and flowers were assessed for the presence of aberrations. Some aberrations are shown in Figure 5.2. Small buds (<1 mm), sometimes appearing as a white dot in axil of the bract are designated as aborted buds according to Roh (1990) (Figure 5.2A). Flowers with deformed tepals and stamens or a deviant number of tepals were designated as aberrant (Figure 5.2B). Stalks with several aberrant leaves (Figure 5.2C) were found in the course of storage and eventually some of the bulbs showed an arrested growth of the shoot (aborted shoot).

In year I, the first aborted buds were observed at 2 °C and at -1 °C after 6 weeks of storage (Table 5.1). They were mostly found in bulbs stored at -4 °C. The number of aborted buds increased in the course of the storage at -4 °C (Table 5.1). In both years, the first aberrant flowers were observed after 12 weeks of storage. In year I, they emerged mainly after 19 weeks of storage, independent of the preceded storage temperature sequence. In year II they were mostly found in plants of bulbs frozen late or at -4 °C. Leaf aberrations were mainly found in plants of deeply frozen bulbs. In year I, after 19 weeks of storage at -4 °C, 17 out of the planted 20 bulbs showed leaf aberrations. In year II, they emerged first after 22 weeks of storage. After 29 weeks of storage seven stalks did not emerge at all or remained small and without flowers. From the 13 remaining stalks 6 showed leaf aberrations. Finally, the stalks with one or more aberrations (aborted buds, aberrant flowers etc.) were counted. The most aberrant stalks were found as result of storage at -4 °C. After 19 weeks of storage in year I all the stalks were found aberrant. In year II after 29 weeks of storage, 14 out of 20 stalks were aberrant (Table 5.1).

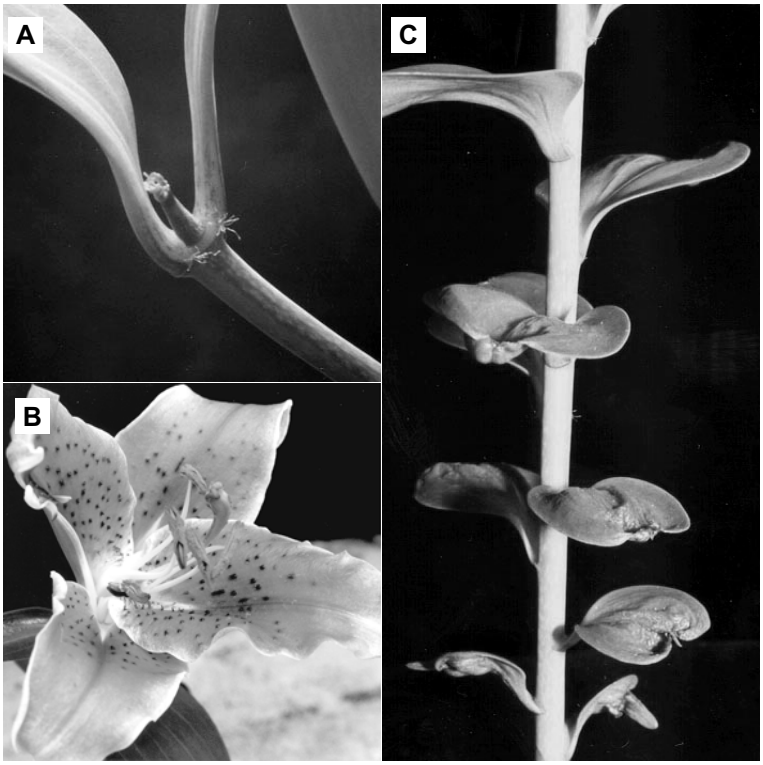

Figure 5.2
Photograph of an aborted bud (A), a flower with a deviant number of aberrant tepals (B) and a stalk with aberrant leaves (C).

Growth of the shoot during storage

A storage temperature of -1 °C did not arrest growth of the shoot because during storage in year I the weight of the shoot increased both in the control and in the late frozen bulbs. Under these conditions growth was not observed in year II. In both years growth was arrested at -4 °C (data not shown).

Water content and water potential

During storage the water contents of the scale and shoot hardly altered and amounted to approximately 63 and 76%, respectively. Except in year I, the shoots of the late frozen bulbs had a water content of about 84%.

The water potential and water content in the basal plate were not affected due to the prolonged storage at -4 °C (Table 5.2). However, the water potential and water content of the shoot apex were lower than that of control bulbs were (Table 5.2).

Table 5.2
Water potential and water content (mean ± standard deviation) of the basal plate and the shoot apex of control bulbs and bulbs frozen deeply measured at 35 weeks of storage (year II) (n=5).

tissue	treatment	sampling point	water potential (MPa)	water content (%)
basal plate	control	(35 wk -1)	-1.4 ± 0.2	72 ± 1.3
	deeply	(6 wk -1) + (29 wk -4)	-1.3 ± 0.2	72 ± 0.9
shoot apex	control	(35 wk -1)	-0.9 ± 0.2	81 ± 1.9
	deeply	(6 wk -1) + (29 wk -4)	-1.4 ± 0.1	74 ± 1.6

Osmolality

In year II, the osmolality of press saps of the shoots of control bulbs decreased from the beginning until 22 weeks of storage at -1 °C (Figure 5.3). The osmolality of bulbs frozen late decreased further than that of control bulbs during the first 6 weeks of storage. During the first 16 weeks of storage at -4 °C the osmolality of the sap of the shoot slightly increased. In year I, the same pattern of changes in the osmolality was found as for year II, but the examined storage period was shorter (Figure 5.3). The osmolality of the scale sap could not be determined because it appeared too viscous.

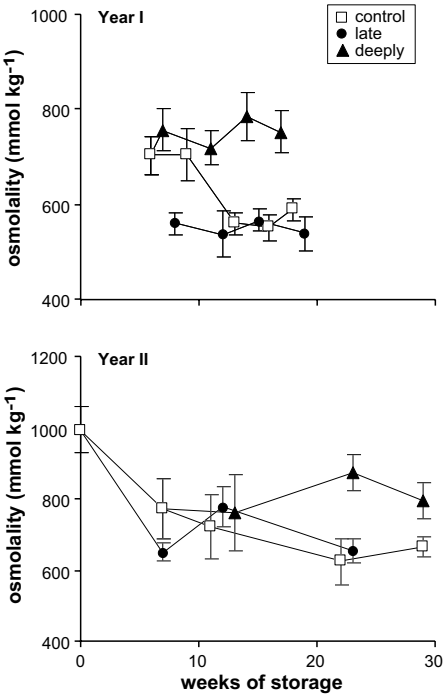

Figure 5.3
Mean osmolality of the shoot of control, late and deeply frozen bulbs (n=10) during storage in year I and II. Error bars represent standard deviations.

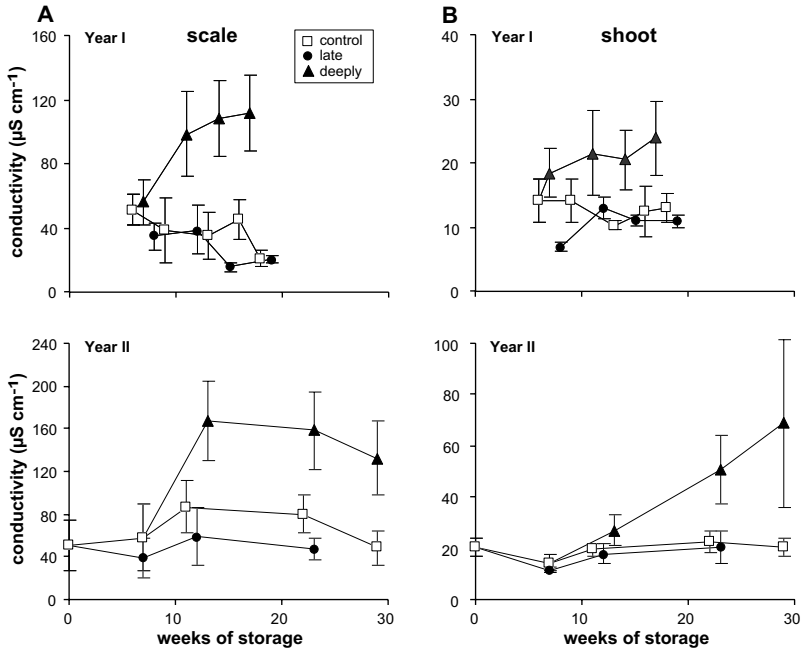

Figure 5.4
Mean conductivity values of the scale (A) and shoot (B) of control, late and deeply frozen bulbs (n=10) during storage in year I and II. Error bars represent standard deviations.

Ion-leakage

In year II, the conductivity of the incubation medium of the scales from control bulbs increased slightly until 22 weeks of storage (Figure 5.4A). The ion-leakage from scales of late frozen bulbs remained essentially constant. As a result of freezing at -4 °C, the ion-leakage from the scales increased but remained essentially constant after 13 weeks of storage. In the shoots of bulbs that were stored at -4 °C a large increase in ion-leakage was found (Figure 5.4B), while the ion-leakage from the shoots of control and late frozen bulbs hardly changed.

In year I the same changes in ion-leakage were found for the scale as well as the shoot tissue (Figure 5.4A,B). Especially in the first period of storage at -4 °C the ion-leakage of the scale increased. The ion-leakage of the shoot of deeply frozen bulbs in year I was also higher than that of control bulbs. Again it should be stressed that in year II storage was continued for a longer time.

T_1 and T_2 relaxation times

At 29 weeks of storage (year II) T_1 and T_2 relaxation times were determined within control bulbs and bulbs frozen late and deeply. The assessed tissues inside the bulbs are indicated in Figure 5.5. The T_1 within deeply frozen bulbs was

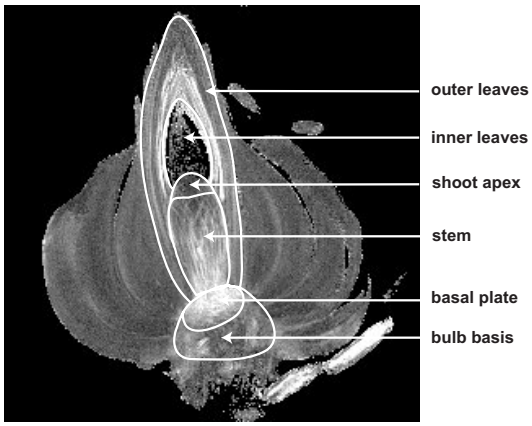

Figure 5.5
T_2-weighted image of a lily bulb with the regions of interest used for the evaluation of the T_1 and T_2 values.

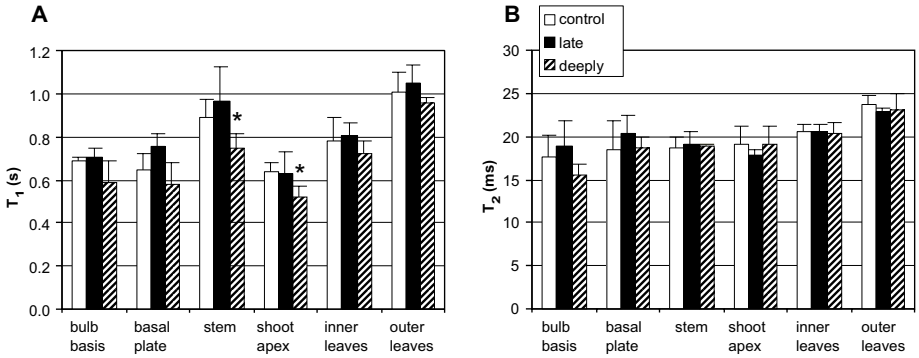

Figure 5.6
Mean T_1 (A) and T_2 (B) values within the different tissues as indicated in Figure 5.5 of control, late and deeply frozen bulbs (n=3) at 29 weeks of storage in year II. Error bars represent the standard deviations. * Significantly different from control bulbs (P<0.05)

significantly lower in the stem and shoot apex than in control bulbs. In late frozen bulbs the T_1 was not significantly different from that in control bulbs (Figure 5.6A). The different temperature sequences had no significant effect on the T_2 in the different ROI's (Figure 5.6B).

Discussion

For the year-round production of lily flowers, the harvest time, the temperature sequence during storage at low and subzero temperatures, and the planting conditions must be chosen properly. The optimal harvest time is set by the physiological state of the bulb, which is dependent on the weather conditions during the preceding season (Gude *et al.*, 2000b). The physiological state at harvest influences the duration of the precooling period required and thus the

timing of the start of the storage at subzero temperatures and the freezing hardiness (Kok *et al.*, 2001). Subsequently, light and temperature during forcing in the greenhouse are relevant for the eventual flower quality. Their effects have been extensively studied (Erwin and Heins, 1990; Treder and Kubik, 2000). The present study concerns the effects of the storage at subzero temperatures of Oriental hybrid lily bulbs and the involved freezing injury.

In the two consecutive years, a 6 weeks delay of freezing did not result in aberrant leaves or aborted shoots. These phenomena were found in plants of bulbs stored 19 weeks or longer at -4 °C. Thus, freezing damage only occurred as a result of prolonged storage at -4 °C. Besides aberrant leaves and aborted shoots also aberrant flowers and aborted buds were found after planting of the too deeply frozen 'Star Gazer' bulbs. These aberrations were also found after planting of control and late frozen bulbs, but in lower numbers. Prolongation of storage at subzero temperatures does additionally lead to a decreased average number of flowers per stalk (Table 5.1) (Lee and Roh, 2001; Tammen and Nissen, 1997).

To assess the effects of freezing damage on lily bulb tissue, water content, water potential and NMR relaxation times were determined. The fresh weight and water content of the whole shoot were not altered as a result of freezing damage. The decreased water content and water potential within the shoot apex of bulbs frozen at -4 °C point towards desiccation of the tissue due to freezing damage. The basal plate appeared unaffected, while in hyacinth bulbs this tissue was reported to be very sensitive to low freezing temperatures (Van der Valk, 1971). Changes in T_1, and especially in T_2 were expected as result of freezing damage. Formerly, longitudinal relaxation times were found to be decreased as a result of freeze-thawing of blueberries and azalea buds (Gamble, 1994; Kaku *et al.*, 1985). T_1 relaxation times were indeed significantly lower in the stem and shoot apex of freezing damaged lily bulbs, possibly as a result of the dehydration of the tissue. Freezing injury is also expected to cause membrane damage, leading to a loss of compartmentalization resulting in an increased T_2 as found in courgettes, carrots and winter wheat (Duce *et al.*, 1992; Hills and Nott, 1999; Millard *et al.*, 1995). Changes in T_2 were, however, not observed but only a small number of bulbs was examined.

In lily bulbs freezing injury was previously related to the sugar content within the bulb. During the pretreatment at low temperatures freezing hardiness was increased due to an increased sugar content (Gude *et al.*, 2000a; Kok *et al.*, 2001). During the storage at subzero temperatures the sugar content subsequently decreased (Kok *et al.*, 2001). In the presented study the osmolality values of the shoot tissue sap, mainly consisting of sugars, also decreased during storage at -1 °C (Figure 5.3). This did not occur at -4 °C. Thus during frozen storage, freezing hardiness appeared higher at lower

osmolality. Yet, the osmolality of the shoot did not increase as a result of prolonged storage at -4 °C, while freezing damage did.

The ion-leakage from the shoot did increase as a result of prolonged too deeply freezing of the bulbs (Figure 5.4B), which may be due to an increased membrane permeability. A direct correlation between the measured parameters and plant quality can not be ascertained. The changes in the measured parameters could in principle also be related to acclimation to lower storage temperatures. It has been suggested, for example, that ion-leakage indicates changes in membrane permeability due to temperature changes. These changes can be reversed when the temperature is increased (Boorse *et al.*, 1998). The increase in the ion-leakage from the scales observed directly after transferring the bulbs to -4 °C was followed by a constant level of ion-leakage (Figure 5.4A). This indeed points towards acclimation to the applied temperatures. However, the continuous increase in the ion-leakage of the shoot is probably more indicative of increasing damage. The large variation in the obtained data may well reflect the variation in the degree of damage. This would indicate that the freezing tolerance of the scales is different from that of the shoot.

Although the changes in parameters like ion-leakage and osmolality in the course of the storage period were similar, the absolute values showed differences between the two assessed years. This is probably due to a combined effect of differences in the preseasonal weather conditions, the harvesting time and the pretreatment temperature.

In summary, the subzero storage of the Oriental hybrid 'Star Gazer' could be delayed with six weeks without the emergence of freezing injury. Prolonged storage at -4 °C did lead to aberrant leaves and aborted shoots. The water content, water potential, T_1 relaxation time of the shoot apex and the ion-leakage of the shoot probably reflect the processes involved in freezing damage.

Acknowledgements

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Addendum to chapter 5

Injuries in Oriental hybrid lily bulbs due to water-soaking

In the preceding part of this chapter, experiments are described in which lily bulbs cv. Star Gazer were deliberately stored at too low temperatures to induce freezing damage. In the first experiment (year I), the bulbs only incurred moderate damage. To further assess the chosen parameters to their ability to indicate damage in stored lily bulbs a batch of lily bulbs was used that already incurred damage. In this part of the chapter, experiments are described with bulbs of the Oriental hybrid *Lilium* cv. Bellezza, obtained from a commercial grower, which were accidentally water-soaked and exhibited severe damage. A similar batch of non-soaked, control bulbs was also obtained. The precise history of the bulbs is unknown.

Because of the small number of available bulbs, it was decided to assess their condition only on the basis of water content, water potential and NMR relaxation times (for Material and methods see main text). The damaged bulbs, and especially the shoots, were soft and brown-colored. The shoot apex and the inner leaves were severely damaged. These tissues were decomposing and could not be dissected. This indicates that in this batch of lily bulbs secondary decaying processes were already occurring.

Table 5.3
Water potential and water content (mean ± standard deviation) of tissues within the water-soaked (n=4) and non-soaked, control (n=5) Oriental hybrid lilies cv. Bellezza.

tissue	treatment	water potential (MPa)	water content (%)
scale	control	-1.7 ± 0.2	60 ± 0.6
	water-soaked	-1.4 ± 0.2	61 ± 1.0
basal plate	control	-1.2 ± 0.2	75 ± 1.1
	water-soaked	-0.6 ± 0.2	82 ± 4.7
stem	control	-1.3 ± 0.1	78 ± 2.4
	water-soaked	-0.6 ± 0.1	83 ± 1.7

The water potential and water content in the basal plate and stem of the injured bulbs were higher than in the same tissues of non-soaked, control bulbs (Table 5.3). Significantly higher T_1 and T_2 values were found in most of the damaged tissues compared to values in control bulbs (Figure 5.7). The increased water content and loss of tissue structure probably contribute to the observed changes in the relaxation times (Du Chatenet *et al.*, 2000).

Compared to the freezing injury, however, the assessed parameters changed differently. The water potential, water content and relaxation times were decreased in the freezing injured tissues. Thus water-soaking had caused damage in more tissues and different processes were involved than in freezing injured bulbs. However, these differences could also be the result of secondary decaying processes active in the extremely damaged 'Bellezza' bulbs. The differences in the T_1 values of healthy 'Star Gazer' and 'Bellezza' bulbs indicate large cultivar differences.

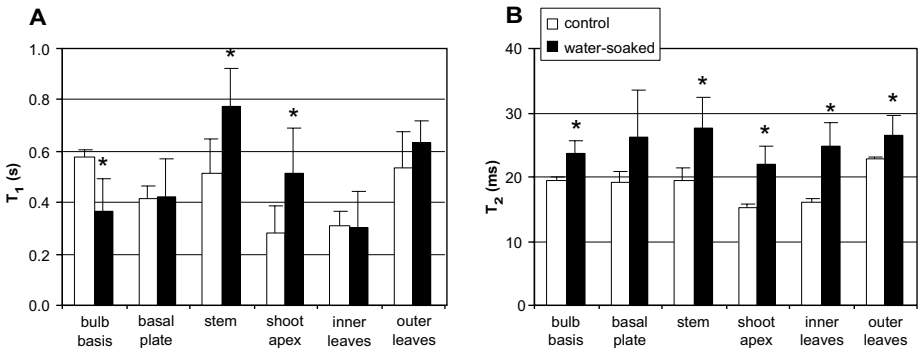

Figure 5.7
Mean T_1 (A) and T_2 (B) values within the different tissues as indicated in Figure 5.5 of water-soaked (n=5) and non-soaked, control (n=4) 'Bellezza' bulbs. Error bars represent standard deviations.
*** Significantly different from control bulbs (P<0.05)**

In summary, the presented, preliminary data indicate that water content, water potential and T_1 (and T_2) relaxation times can be used to distinguish different kinds of damage in lily bulbs.

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Chapter 6

Physiological disorders in stored flower bulbs assessed by magnetic resonance imaging

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Submitted

Abstract

In horticultural practice, the forcing of flower bulbs may induce physiological disorders, which are usually not visible externally. The possibilities of magnetic resonance imaging (MRI) as a tool to detect such disorders induced by storage conditions have been assessed. With the use of T_2-weighted images, water concentration maps and T_1 and T_2 maps, both bud abortion in *Hippeastrum* and tulip bulbs and water-soaking in lily bulbs could be detected and studied.

Introduction

For the year-round production of cut flowers, flower bulbs are usually stored at different temperatures to control flower development after planting. Unfortunately, these forcing procedures can also result in physiological disorders or an increased sensitivity to such disorders. Damage induced by storage conditions is often not visible externally and remains usually undetected until planting. Thus, the assessment of the internal quality of stored flower bulbs is an important issue in horticultural practice.

The physiological disorders sometimes involve morphological changes. Very often the aberrations are accompanied by impairments in the water status of the bulb. In both cases, magnetic resonance imaging (MRI) can provide a non-invasive tool to monitor developmental abnormalities. MRI can visualize the distribution and motional properties of water molecules and therefore distinguish tissues inside a plant and indicate changes in the water status.

The intensity of the signal measured by MRI is dependent on the number of water molecules present. The local water concentration can be measured from the signal intensity. The motional properties of the water molecules, are described by the longitudinal (T_1) and transverse (T_2) relaxation times. Mobile water is characterized by relatively high values of T_1 and T_2, while shorter values are indicative of stronger interactions of water with the surrounding macromolecules and solutes (Bendel *et al.*, 2001). Because plant cells and even cell compartments have a different chemical composition, T_1 and T_2 relaxation times will vary throughout the plant. Transverse relaxation (T_2) is also influenced by the diffusion of water through different local magnetic fields induced by the different cell compartments and by air-filled extracellular spaces (Köckenberger, 2001). This implies that MRI may reflect biological processes that involve for example changes in the composition of cell saps or changes in compartmentalization, including loss of membrane integrity.

In the present study MRI was used to assess some physiological disorders in flower bulbs. Bud abortion, i.e. the failure of a bulb to produce a marketable flower after the floral organs have been formed (De Hertogh and Le Nard, 1993) is a frequently occurring physiological disorder in flower bulbs. The disorder is often induced during storage but usually manifests after planting. Bud abortion is a large problem in the forcing of *Hippeastrum* (amaryllis) bulbs. The causes of this disorder are still unknown. Bud abortion can be easily detected in *Hippeastrum* bulbs by cutting the bulbs. However, these bulbs are relatively expensive and a single bulb can produce flowers for many years. The possibilities to detect the aborted buds in *Hippeastrum* bulbs with the use of

MRI were therefore assessed. Bud abortion in tulip bulbs can be induced by several forcing conditions and manifests as a small, desiccated bud after planting (Chapter 3). In severe cases the whole shoot does not emerge at all after planting (shoot abortion). Desiccation of the flower bud is expected to be the final result of a change in the water status (Chapter 2). MRI was used to study local changes in the water concentration within tulip bulbs. Bud abortion was induced by long-term cold and dry storage.

A different kind of disorders encounters during the forcing of lily bulbs. Harvesting of lily bulbs takes place in late autumn. Excessive rainfall in the fall can obstruct the harvesting, resulting in water-soaked bulbs. Freezing injuries in lily bulbs as a result of long-term frozen storage for the year-round production of flowers, can also reduce the flower quality. Freezing injuries and water-soaking often have an effect on the structure of the tissue and the properties of tissue water. Thus, it was hypothesized that T_1 and T_2 relaxation times determined by MRI can discriminate between normal and damaged tissue. This was tested in water-soaked lily bulbs.

Materials and methods

Measurements were performed on a 4.7 T (200 MHz) NMR instrument (Varian, Palo Alto, CA) using a volume coil. A Hahn spin-echo pulse sequence was used with the following parameters: Field of View (FOV) of 6x6 cm^2, 256x256 matrix, 2.5 mm slice thickness, and two transients.

T_2-weighted images were used for the detection of morphological changes. *Hippeastrum* 'Orange Souvereign' bulbs were imaged with an echo time (TE) of 4.8 ms and repetition time (TR) of 5 s.

Water concentration maps of tulip bulbs (*Tulipa gesneriana* L. 'Apeldoorn'), stored at 5 °C for 35 weeks, were calculated from a series of T_2-weighted images with TE's of 4.8, 5.5, 8, 15, and 30 ms (TR, 5.1 s) by extrapolating TE to 0 ms and correcting this proton density map for B_1-field inhomogeneities and the signal intensity of an external concentration standard (capillary).

T_2-maps of water-soaked and non-soaked, control lily bulbs (*Lilium* Oriental hybrid 'Bellezza'), were calculated from a series of T_2-weighted images with TE's of 4.8, 5.5, 8, 15, and 30 ms (TR, 5.1 s). T_1-maps were calculated from a series of T_1-weighted images obtained with an inversion recovery pulse sequence with inversion times of 0.0007, 0.1, 0.5, 1 and 5 s (TE, 4.8 ms; TR, 5.1-10.1 s).

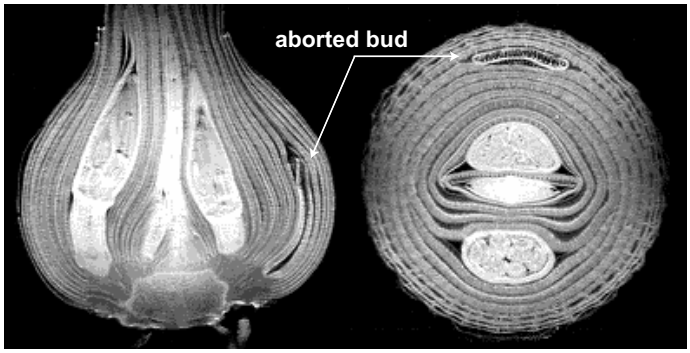

Figure 6.1
T_2-weighted longitudinal and transversal image of a *Hippeastrum* (amaryllis) bulb 'Orange Souvereign' showing an aborted bud.

Figure 6.2
Water concentration maps of tulip bulbs at 12, 23 and 35 weeks of cold, dry-storage, resulting in bud abortion after 23 weeks of storage and subsequent planting. The water concentration in the capillary was 55 mol L^{-1}.

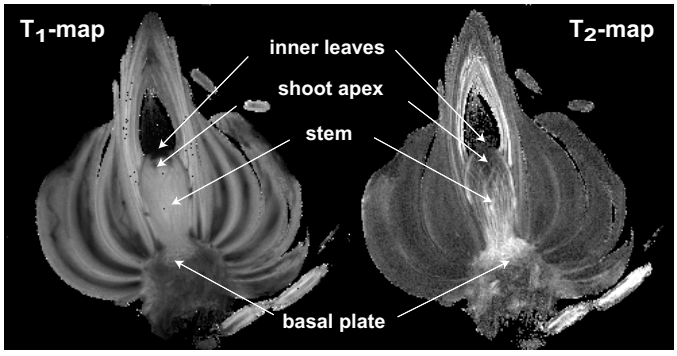

Figure 6.3
T_1 and T_2 maps of a water-soaked bulb of the Oriental lily hybrid 'Bellezza'. Bright areas represent long relaxation times, dark areas short times. The shoot apex and the inner leaves appear affected.

Table 6.1
Quantified T_1 and T_2 values (mean ± standard deviation) of different tissues within non-soaked, control (n=4) and water-soaked (n=5) bulbs of the Oriental lily hybrid 'Bellezza'.

	T_1 (s)		T_2 (ms)	
tissues	control	water-soaked	control	water-soaked
basal plate	0.42 ± 0.05	0.41 ± 0.15	19 ± 2	26 ± 8
stem	0.51 ± 0.13	0.80 ± 0.15 *	19 ± 2	28 ± 4 *
shoot apex	0.28 ± 0.10	0.55 ± 0.23 *	15 ± 1	23 ± 3 *
inner leaves	0.31 ± 0.05	0.33 ± 0.17	16 ± 1	25 ± 4 *

* Significantly different from normal bulbs (P<0.05)

Results and discussion

In *Hippeastrum* bulbs, inflorescence initiation alternates with scale formation throughout the entire growth period. It is evident from the images (Figure 6.1) that after every four scales (developing into leaves) a floral stalk with a number of flowers is developed. The presence of aborted buds in *Hippeastrum* bulbs can be observed clearly in longitudinal and transversal T_2-weighted images (Figure 6.1).

Bud abortion in tulip bulbs was observed after 23 weeks of cold and dry storage and subsequent planting. During storage, a gradual decrease of the water concentration in the shoot was found (Figure 6.2). The dehydration probably reflects an increased risk of bud abortion after planting. Furthermore, a drop in the water concentration of the stamens during storage (clearly visible at 35 weeks) probably indicates the withdrawal of water from the shoot to the enlarged daughter bulb, resulting in shoot abortion (Chapter 3).

The motional properties of water molecules in lily bulbs were expected to be changed as a result of water-soaking. T_1 and T_2 relaxation maps of water-soaked bulbs were calculated and compared to those of non-soaked, control bulbs. A T_1 and T_2 map of a water-soaked bulb is depicted in Figure 6.3. The differences in contrast within the two maps are remarkable. Furthermore, T_1 and T_2 were increased in most tissues due to the induced damage (Table 6.1). Apparently, a change in the overall mobility of the tissue water had occurred due to water-soaking. The cell membranes are probably damaged, resulting in a decreased diffusional barrier and increased T_2 relaxation times (Millard *et al.*, 1995). The filling of air-spaces in the tissue due to membrane damage may also contribute to the increased T_2 relaxation times (Duce *et al.*, 1992).

In summary, MRI can be used to detect non-invasively physiological disorders in flower bulbs. Morphological changes and changes in the water status can be reflected by the water concentration values and the T_1 and T_2

relaxation times. MRI can also be used to assess physiological disorders and changes in maturity in fruits and vegetables (Clark *et al.*, 1997) as well as root and shoot growth of potted plants (Chudek and Hunter, 1997; Faust *et al.*, 1997). In contrast to other non-destructive detection techniques, like near infrared spectroscopy, an image plane within the (core of the) object can be chosen optionally and contrast can be manipulated according to the MRI parameters chosen (Clark *et al.*, 1997). Unfortunately, the MR-imager is an expensive instrument. However, the possibilities of MRI to assess the internal quality of horticultural products are promising and on-line applications will become more feasible in the future.

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Chapter 7

General discussion

Throughout the years, empirical research on flower quality after planting has led to the most optimal storage and planting conditions for flower bulbs (Hoogeterp, 1973). The underlying physiological processes are, however, not always clear (Hartsema, 1961; Rees, 1969). To accelerate or to delay the development of the bulb for the production of year-round bulbs and cut flowers, sub-optimal storage conditions are often applied, which may lead to a variety of disorders in the development of the bulb, as for instance bud abortion and leaf aberrations. The effects of the applied forcing conditions are usually not visible externally. They become apparent at flowering, even though they are often induced during storage. An early detection of these disorders and understanding of the factors and processes involved in their development are thus important subjects of research for horticultural practice. Changes in the water status are expected to be often involved in the development of the induced aberrations. Hence, in this thesis the effects of the applied storage conditions on the internal quality of flower bulbs were assessed by studying different water status parameters.

The physiology of flower bulbs during storage

Cold fulfillment

For the production of flowers early in the season, bulb development must be accelerated artificially. Many bulbs require a minimal period of low temperatures to enable root and shoot development (Le Nard and De Hertogh, 1993a). If this period of cold is applied too soon or not long enough, aberrations like bud abortion in tulip can occur after planting (De Munk and Hoogeterp, 1975).

Especially in tulip the cold requirement has been studied frequently (Le Nard and De Hertogh, 1993b). The duration of the cold storage is cultivar specific. Tulip bulbs cv. Apeldoorn are dry-stored at 5 °C for 12 weeks to ensure optimal flowering when planted at higher temperatures in the greenhouse (Boonekamp *et al.*, 1990; Moe and Wickstrøm, 1973). Research has been done for a number of years, in order to understand the processes underlying the cold requirement and to find indicators for its proper fulfillment (Boonekamp *et al.*, 1990). Hormones like gibberellins and cytokinins in combination with auxins may play a role since they can partly replace the cold requirement in tulip bulbs (Saniewski and Kawa-Miszczak, 1992). During storage at low temperatures starch is converted into sucrose (Moe and Wickstrøm, 1973). It has been suggested that hormones may play a role in the sink source relations between the scale and the shoot (De Munk, 1979) and consequently affect the transport of sucrose plus water to the shoot and developing daughter bulbs. Since the bulbs are dry-stored, growth of the shoot occurs at the expense of internal water, stored in the scales. Accordingly, it may be anticipated that the osmotic potential and the water status alter during the cold fulfillment.

In the present study, a rapid increase of the osmolality in the saps of the scales and shoots was found as a result of dry-storage at low temperatures (Chapter 2 and 3). In non-cooled bulbs, which are continuously stored at moderate temperatures, the increase in the scales was less prominent (Chapter 4). Bendel *et al.* (2001) suggested that the increased sugar concentration in tulip bulbs at low temperatures might result in an increased influx of water molecules from outside the cells to intracellular compartments, which correspondingly could be reflected in changes in the motional properties of water determined by magnetic resonance imaging. They further suggested that with the conversion of starch into sugars, the motionally restricted and partially ordered water pool inside the starch grains might be released (Bendel *et al.*, 2001). Both effects would involve changes in the mobile water pool and accordingly affect the longitudinal (T_1) and transverse (T_2) relaxation times in the bulb. Van der Toorn *et al.* (2000) found that throughout storage at low

temperatures, T_1, T_2 and proton density in the basal plate decreased further than at moderate storage temperatures. In their study, the proton density and T_2 in the scales hardly changed until 8 weeks of storage. Subsequently, the T_2 in the scales of cooled and non-cooled bulbs decreased. In this study (Chapter 2), T_1 and T_2 in the basal plate also decreased throughout storage, while in the scales no significant changes were observed. The T_1 and T_2 in the shoot decreased strongly during the first 12 weeks of storage (Chapter 2 and 3). NMR data from non-cooled bulbs are lacking. The decreases in the relaxation times suggest a decreased mobility of the water molecules during the fulfillment of the cold requirement in tulip bulbs. This may partially be related to an increased sugar concentration and the associated increased viscosity (Chapter 3). The shortened relaxation times in the shoot may also be related to the reconversion of imported sugars into starch (Moe and Wickstrøm, 1979).

Aside from assessing the water status in cold treated tulip bulbs using MR relaxation parameters, many other attempts have been made to trace indicators for the fulfillment of the cold requirement. Hormonal activity, metabolic activity, membrane physiology and respiratory activities of tulip bulbs during the cold fulfillment have been studied (Kanneworff, 1995; Lambrechts, 1993; Rebers, 1994; Walch, 1997). The ion-leakage test has not been used previously for this particular purpose. The ion-leakage from the scales increased and reached a plateau value after 12 weeks of storage at low temperatures (Chapter 2 and 4). The ion-leakage from non-cooled bulbs increased little during storage. The increase in ion-leakage might be related to the acclimation of the bulb tissue to low temperatures and thus may indirectly be indicative of the fulfillment of the cold requirement. Applying elevated temperatures after the cold fulfillment would provide more information on the temperature dependence of the ion-leakage.

Effects of high storage temperatures

Although flower bulbs often require a definite period of low temperatures for shoot elongation after planting, flower development can be accelerated by applying moderate to high temperatures directly after harvesting (Hoogeterp, 1973; Slootweg and Hoogeterp, 1971). However, when the high storage temperatures are not directly applied after harvest, flower development can be impaired and instead growth of daughter bulbs is promoted after planting (Rees, 1967). During storage at high temperatures, the water status remained unchanged, since most of the water status parameters were unaltered (Chapter 4). Nevertheless, flower development was abnormal and thus other factors, like the hormonal status, may play a role. For example ethylene is known to abort the development of the shoot and to stimulate daughter bulb development when applied in the proper doses and at the appropriate time (De Munk and

Gijzenberg, 1977). Furthermore, Kanneworff and Van der Plas (1994) related an increased endogenous ethylene level to an increased risk of bud abortion. Unfortunately, it remains unclear whether ethylene exposure induces similar processes as storage at high temperatures does (Chapter 4). The ethylene induced bud abortion as described by De Munk (1973) mostly resembles the morphological changes that were found after long-term cold storage (Chapter 2 and 3). These aberrations were different from those found after storage at high temperatures.

Effects of long-term cold and dry storage

Not only storage at high temperatures but also long-term dry storage at low temperatures can lead to a reduced flower quality or even to bud and shoot abortion. The phenomenon has hardly been studied and literature on the physiological effects of long-term storage on flower bulbs is thus scarce. The effects of the long-term storage on the flower quality may be related to an impaired water status. In tulips dehydration of the shoot appears to be involved in the processes leading to bud abortion as induced by long-term cold (5 °C) and dry storage of the bulb (Chapter 2 and 3). During storage at low temperatures, bulbs are not fully in rest (Chapter 2 and 3). Growth and development continue even at subzero temperatures (Chapter 5) (Miller and Langhans, 1990). Water is used continuously for growth, while it additionally evaporates from the bulb, especially during dry storage. The dehydration of the shoot might be worsened by an impaired development of the roots after planting as a result of desiccation or high soil temperatures, leading to a reduced water uptake. A shortage of water and metabolites in the shoot may additionally be caused by premature growth of the daughter bulbs. During long-term cold and dry storage (Chapter 2 and 3) the daughter bulbs grew considerably. Under natural circumstances growth of the daughter bulbs occurs during planting when the roots and leaves can supply the "extra" water and metabolites required (Le Nard and De Hertogh, 1993b). The question remains whether the growth of the daughter bulbs was induced as a survival mechanism when chances on generative propagation were minimized due to desiccation of the bud, or whether the growth of the daughter bulbs actually caused the water deficit of the shoot.

Effects of frozen storage

Storage of tulip bulbs at subzero temperatures does also reduce the flower quality. Thawing and refreezing results in damage to the crop. It is thought that due to the accelerated development during the thawing period, the bulb reaches a stage at which it becomes intolerant to refreezing. In a preliminary study on bud abortion in 'ice-tulips', bulbs were packed in moist soil, stored

at subzero temperatures, left to thaw and transferred back to freezing temperatures. Directly after thawing or just before refreezing the bulbs were repacked in moist soil. Finally the bulbs were planted. Thawing resulted in bud abortion while refreezing did not lead to additional damage. The number of aborted buds appeared to be related to the moisture level in the soil during the thawing period (unpublished results P. Tersteeg). These data indicate that a water deficit, and thus the availability of water for growth during the fast development of the roots and shoot during the thawing period probably causes the damage to the crop, while refreezing is of minor importance.

Dehydration of tissues also occurred during storage of lily bulbs when too low freezing temperatures were applied resulting in freezing injury. Freezing injury coincided with a decreased water content in the shoot apex (Chapter 5). However, a continuous decrease of the water content of the shoot, as found during long-term cold and dry storage of tulip bulbs was not observed. Freezing injury due to the application of too low storage temperatures did not occur directly at the beginning of storage but occurred several weeks later. This could imply that during storage, the shoot became less tolerant to the applied temperatures or that tissue damage spread slowly throughout the bulb, remaining undetected initially.

When the start of the storage of lily bulbs at freezing temperatures was delayed, freezing tolerance was expected to be reduced. This may happen because the shoot reaches a freezing intolerant physiological state, which is related to the internal sugar concentration (Kok *et al.*, 2001). Unfortunately, in the present study on the delayed application of freezing temperatures damage to the flower buds was not observed (Chapter 5), possibly because the applied six weeks delay was too short. The initiation and formation of the flower buds in lily bulbs can vary within cultivars and harvest (Kok *et al.*, 2001; Wilkins and Dole, 1997). Therefore it would be interesting to know whether freezing tolerance, aside from sugar concentration, also depends on the developmental stage of the flower buds.

Effects of greenhouse conditions

Aside from the physiological state at harvesting and the subsequently applied storage condition, sub-optimal planting conditions can also have an unfavorable influence on the flower quality. Depending on the history of the bulb, planting conditions have different effects, which also might involve changes in the water status of the plant. Impairments can sometimes be minimized (Chapter 3) and sometimes enhanced during planting (Lee and Roh, 2001). Light deficiency or high soil temperatures for example can be the death-blow to a weakened bulb (Le Nard and De Hertogh, 1993b; Wilkins and Dole, 1997). Sub-optimal planting conditions can result in similar symptoms as found after sub-optimal

storage conditions. For example, bud abortion in tulip bulbs can be induced by storage as well as planting conditions (De Munk and Hoogeterp, 1975; Le Nard and De Hertogh, 1993b). Therefore, it is sometimes difficult to determine, based on the phenomena found after planting, which processes contributed to the disordered development of the plant and when these occurred. Thus, the understanding of the processes underlying the aberrations induced by sub-optimal storage conditions requires the detection of these aberrations at an early stage of development.

The water status of flower bulbs during storage

'Classical', invasive methods have been used to indicate changes in the water status of tulip and lily bulbs during the fulfillment of the cold requirement as well as during the induction of several disorders. These results are summarized in Table 7.1.

Dehydration of tissues within the assessed tulip and lily bulbs was indicated by the water content, water concentration and water potential. These parameters decreased similarly during long-term cold and dry storage of tulip bulbs and during storage of lily bulbs at subzero temperatures (Chapter 3 and 5, Table 7.1). In water-soaked lily bulbs, both the water content and the water potential were increased (Addendum to chapter 5, Table 7.1). Changes in the water content were particularly evident in the stamens of tulip and the shoot apex of lily. Apparently these tissues are sensitive to water stress (Chapter 3 and 5, Table 7.1). T_1 and T_2 relaxation times were often indicative of impairments in the water status. The course of the changes in these parameters was not related to changes in the water content, water potential or osmolality (Chapter 3).

In summary, the water status was always altered as a result of the applied storage conditions, except for storage at high temperatures (Table 7.1). In the latter case, the water status hardly changed (Chapter 4).

Enhanced ion leakage from plant tissues has often been used as a viability test, amongst others in case of freezing injury (Bonnier *et al.*, 1994; McCollum and McDonald, 1991; Stergios and Howell Jr, 1973). It has been suggested that the ion-leakage reflects changes in membrane permeability and consequently might have an effect on the transmembrane mobility of water molecules. Thus, ion-leakage can reflect changes in the water status and/or viability of tissues.

Freezing injured lily bulbs as well as tulip shoots after storage at high temperatures showed indeed an increased ion-leakage (Chapter 4 and 5, Table 7.1) The ion-leakage from the scales of tulips increased, however, also during

Table 7.1
Changes found in the various water status parameters as a result of the applied storage conditions. + = change, - = no change, nd = not determined.

species	developmental process	water content		osmotic potential	water potential	T_1 and T_2		ion-leakage
		scale/ shoot	stamens/ shoot apex	scale/ shoot	stamens/ shoot apex	scale/ shoot	stamens/ shoot apex	scale/ shoot
tulip	cold requirement	-	nd	+	nd	+	+	+
	bud abortion due to long-term cold storage	+	+	+	+	+	+	-
	bud abortion due to high storage temp	-	nd	-	nd	-	-	+
lily	freezing injury	-	+	+	+	±[a]	±[a]	+
	water-soaking	nd	+	nd	+	+	+	nd

[a] **significant differences in T_1, no differences in T_2**

the cold fulfillment (Chapter 2, Table 7.1). During long-term cold storage of tulip bulbs a decrease in viability and therefore an increased ion-leakage was expected, but not found (Chapter 2, Table 7.1). Boorse *et al.* (1998) proposed that ion-leakage cannot be used as a direct measure of cell death. The leakage of ions reflects changes in the plasma membrane permeability as acclimation to temperature changes and should therefore be reversible. Acclimation to temperature changes appeared to be involved in scales of lily bulbs after the transfer to lower freezing temperatures (Chapter 5). However, after storage at high temperatures and subsequently applied low temperatures, ion-leakage from damaged bulbs remained higher than from normal ones (Chapter 4). So, in bulb tissue ion-leakage appears to reflect both acclimation to temperature changes and changes in viability.

MRI in flower bulb research

The water status of tulip and lily bulbs during storage at optimal and sub-optimal conditions was ascertained by 'classical', invasive methods. They provide a valuable tool for the assessment of the internal quality of flower bulbs. The possibilities of magnetic resonance imaging to assess non-invasively the internal quality of flower bulbs are discussed below. With the use of MRI, different information on the water status can be obtained from macroscopic to almost microscopic level throughout developmental processes. Regions of interest can be chosen after completing the measurements.

T_2-weighted imaging appeared to be a suitable application to visualize the morphological changes in bulbs like growth of the shoot and the

development of daughter bulbs (Chapter 2 and 3). Aberrant developments of the floral parts in tulip bulbs after storage at high temperatures and the presence of aborted buds in *Hippeastrum* bulbs could well be observed (Chapter 4 and 6). Water concentration and relaxation parameters were successfully used to assess the water status in the bulbs and the water status related disorders like bud abortion in tulips after long-term cold and dry storage and freezing injury and water-soaking in lily bulbs (Chapter 2,3,5,6).

Considerations to MRI in flower bulb research

As shown in the present study, T_1 and T_2 in tulip and lily bulbs strongly change during storage at optimal and sub-optimal conditions. The biological meaning of the observed changes, however, is not always clear. While the physical parameters T_1 and T_2, often used as indicators for the water status, can be determined rather easily, many physiological variables influence the value of these parameters.

Longitudinal relaxation (T_1) is not only influenced by the often suggested water content (Chapter 3) (Ratcliffe, 1994; Ruan and Chen, 1998). Measurements in pressed tissue sap of tulip bulbs clearly showed that the composition of the tissue sap strongly affected T_1. The anatomical structure of the tissue had less influence on this parameter (Addendum to chapter 3). The question, which particular components influence the T_1 and what their importance is for the development of the bulbs, requires further study.

The T_2 in the pressed sap was also somewhat affected by the chemical constituents. In contrast to T_1, however, the parameter was also influenced by the anatomical structure of the tissue and the diffusion of water molecules within the tissue (Addendum to chapter 3). Because tulip bulbs contain many starch grains and have small vacuoles, the influence of diffusion within and across different compartments on T_2 cannot easily be extrapolated from model systems (Hills and Snaar, 1992; Snaar and Van As, 1992). The effect of these diffusion processes on T_2 relaxation times depends on the used pulse sequence and timings (Hills and Duce, 1990; Hills and Nott, 1999), resulting in different T_2 values for each experimental set-up. The influences of diffusion can be minimized by using a multi-echo sequence with a short pulse spacing (Duce *et al.*, 1992).

The relatively immobile pool of water is not included in the T_1 and T_2 measurements. These water molecules have a very fast transverse relaxation rate and cannot be determined with a regular spin-echo sequence. Notwithstanding these limitations, T_1 and T_2 relaxation times could be determined reproducibly in tulip bulbs and appeared to be characteristic for the assessed tissues (Chapter 3).

A more straightforwardly interpretable MRI-based water status parameter is the quantified proton density or the water concentration. The MRI data correlated well with water concentrations obtained by weighing (Chapter 3). The values obtained with MRI are underestimated on one hand because the immobile water pool is neglected. On the other hand they are overestimated because signals from other molecules than water (e.g. carbohydrates) are not discriminated as such. But most important, MRI could clearly identify dehydration effects in tulip bulbs (Chapter 3).

The non-invasive nature of MRI makes it suitable for serial studies monitoring responses to particular storage treatments. However, the experimental set-up should be carefully chosen. For example, it cannot be ruled out that the fixation of the bulbs inside the RF-coil slightly damaged the root primordia, resulting in a somewhat decreased root development and consequently a decreased flower quality after planting of the imaged bulbs (Chapter 3). In the experimental set-up, dry air was blown around the fixed bulb to keep it at the required storage temperature. This may have caused some dehydration of the bulbs, especially of those that were studied multiple times (Chapter 2).

Because flower bulb development is very sensitive to the applied temperatures, storage temperatures should be maintained during the measurements. Not only because temperature shifts might be disadvantageous to the bulb, but also because the water status at the applied storage temperatures is of importance. Yet, the measuring-temperatures have a major effect on for example the longitudinal relaxation times (Chapter 4) (Nelson and Tung, 1987). Similar 'technical' problems would also be encountered when other (non-invasive) techniques had been used.

Perspectives of MRI in flower bulb research

In the present study the use of MRI was restricted to measurements in (dry-) stored bulbs. In a scientific setting there are, however, many additional possibilities for this technology. Root and shoot growth in frozen soil can be examined with the use of MRI because the bulbs do not freeze at the usual storage temperatures, e.g. -2 °C. Studies on freezing tolerance and freezing points can be performed by adapting the temperatures inside the magnet. Furthermore, a magnet with a vertical bore allows the study on growth and development of potted bulbs. Root and shoot elongation of potted plants as well as the growth of the daughter bulbs can be examined. Thus, MRI can in principle contribute to many aspects of bulb research.

For agricultural and horticultural applications the feasibility of large-scale/on-line MRI techniques are being explored (Constantinesco *et al.*, 1997; Kim *et al.*, 1999). This opens the possibility of screening large amounts of flower bulbs to select diseased or distorted ones.

Large-scale studies on dry-stored bulbs are most feasible, especially when the (physiological) state, including the presence of aberrations, can be determined at a single time point. The present study shows that MRI in a scientific setting can be used to detect several disorders in flower bulbs. The detection of the disorders was based on an altered morphology (Chapter 4 and 6) or on impairments in the water status (Chapter 2,3,5,6). Below, a few examples are given of possible large-scale applications of MRI to detect aberrations in flower bulbs.

A potential large-scale application of T_2-weighted imaging is the assessment of the flower quality of tulip bulbs after exposure to high storage temperatures (Chapter 4). With a single image of the shoot of a stored bulb, floral aberrations can be visualized. With T_2-weighted imaging the presence and the size of daughter bulbs in cold-stored tulip bulbs can be determined. This gives relevant information on the risks of bud abortion and additionally on the propagation rate of the stock (Chapter 3 and 4). The presence of aborted buds in *Hippeastrum* bulbs can also be detected with the use of T_2-weighted imaging (Chapter 6).

T_2-weighted imaging cannot provide sufficient information with regard to changes in the water status. Such information can be provided by quantitative measurements of T_1 and T_2 relaxation times. Water-soaking and freezing injury in lily bulbs, for example, resulted in changes in the water status, which were reflected by changes in the T_1 and T_2 relaxation times (Chapter 5). T_1 and T_2 measurements showed that during long-term cold storage of tulips the water status of the shoot changed (Chapter 2 and 3). The decline in T_1 and T_2 relaxation times in the shoot below a certain threshold value was suggested to be indicative of an increased risk of bud abortion after planting (Chapter 2). The drop in the water concentration in the stamens indicated severe changes in the water status, which were probably related to shoot abortion (Chapter 3).

Aside from the studied disorders, it is expected that MRI can also be used for on-line detection of other flower bulb disorders and diseases. For instance, T_2-weighted imaging could be used to trace *Fusarium* infected tulip bulbs, which constitute a large problem in tulip bulb breeding. The infection causes widening of the scales, which can be measured as enlarged spaces between the scales.

MRI is increasingly used to assess the internal quality of fruits and vegetables (Chudek and Hunter, 1997; Faust *et al.*, 1997). The technique has

shown to be a useful tool in the assessment of maturity changes and physiological disorders in a wide variety of horticultural products (Chen *et al.*, 1989; Clark *et al.*, 1997; Scheenen *et al.*, 2000).

Although MRI appears to be the method of choice for the detailed study of water distributions in plants, it does not readily provide, at least in the present configuration, possibilities to efficiently screen populations of bulbs. Currently most MRI measurements are time consuming. This can probably be improved with advancing technology and upscaling of the imaging capacity. When accepting T_1-weighting, and thus saturation effects, the acquisition time of the T_2-weighted images for examining anatomical changes can be shortened. To obtain parametric images for information on the water status, a series of images with different delay times is needed and saturation must ideally be prevented. This increases the measurement time considerably. More time-efficient imaging sequences, e.g. a CPMG-like sequence in stead of the used Hahn spin-echo sequence, could be used in case the T_2 relaxation times are not too short (>10 ms). However, the main limitations for the large-scale application of MRI in horticulture are the costs of the implementation of a MRI system. Costs can be somewhat reduced when using low magnetic fields. However, the signal to noise ratio decreases at lower magnetic fields.

Non-invasive alternative methods

To avoid the high costs of MRI, most researchers use alternative non-invasive methods to assess the internal quality of horticultural products (Abbott, 1999; Chaerle and Van der Straeten, 2002; Chen and Sun, 1991). The appearance, release of volatiles, firmness and fluorescence of the fruits or vegetables are characteristics that can be used to assess maturation processes (Ciscato *et al.*, 2001; Mizrach, 2001; Persijn *et al.*, 2001).

Near infrared spectroscopy (NIR) can be used to detect water, carbohydrates, fats and proteins near the surface of the product. Based on the soluble solids, maturity and ripeness can be determined in many fruits. NIR is also used to determine the oil content in seeds, nuts and avocados (Abbott, 1999). NIR is already used commercially to assess the quality of horticultural products (Chen and Sun, 1991).

X-ray transmission depends on the mass density and absorption of the material and correlates to the moisture content (Barcelon *et al.*, 1999; Sonego *et al.*, 1995). Disorders are usually associated with changes in the density of the internal tissue and the distribution of water and can thus be visualized by X-ray. Like MRI, X-ray can be used to detect disorders like water core in apples, split pit in peaches, freezing damage in citrus, the presence of pits in olives and cherries and hollow heart in potatoes (Barcelon *et al.*, 1999). For

the latter, the use of X-ray already is commercial routine. The online, large-scale selection of *Fusarium* infected tulip bulbs has also become possible by X-ray. The infection results in the widening of the bulb scales. The ratio between the area of the tissue and the cavities in between, is used for the selection. X-ray sensors for *Fusarium* infected tulip bulbs with a throughput of 40.000 bulbs per hour, are now in production (Hans Valk, Havatec, personal communication).

Currently most researchers agree that MRI has a great potential for the evaluation of the internal quality of horticultural products but that the technique is still too expensive for routine, high-throughput applications (Clark *et al.*, 1997). However, in the future MRI is expected to become cheaper, faster and more feasible for specialized applications (Abbott, 1999).

In summary, this study has augmented the knowledge of some physiological disorders in flower bulbs induced by sub-optimal storage conditions. MRI can be used to assess the internal quality of flower bulbs, which is often related to their water status. This knowledge may provide flower bulb growers with better tools to evaluate their storage protocols and thus eventually may contribute to cost reduction and quality control.

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Summary

Many flower bulbs have a life cycle of a year or more, flowering either in spring or in summer. Nevertheless, year-round production of cut flowers has become common practice in horticulture. To control flowering, which is necessary for the year-round production of flowers, bulbs are exposed to specific temperature regimes to affect their growth and development. When storage or planting conditions are not properly chosen, physiological disorders in bulb development may occur. Flower bud abortion and aberrant leaves are examples of such physiological disorders. Disorders in the bulbs are usually not visible externally. They become apparent after planting even though they are often induced during storage.

The aim of this study was to gain a better insight in the processes underlying physiological disorders in flower bulbs, induced by improper storage conditions. Changes in the water status are expected to be often involved in the development of the induced aberrations. The water status of stored flower bulbs was therefore assessed using both magnetic resonance imaging (MRI, known from the medical applications) as well as classical water status measurements. It was assessed whether the obtained parameters might serve as 'early indicators' of an impaired flower bulb quality and thus as indicators for the internal quality of stored bulbs. This might lead to a tool to detect the presence of disorders at an early stage of development. Such an application could avoid further cost of storage and planting of impaired bulbs and potentially increases the chances to minimize the damage. Furthermore, it would enable optimization of storage conditions of new flower bulb genera more easily. In addition, flower quality can be better guaranteed.

In this research the mechanism underlying several physiological disorders in flower bulbs was studied by exposing the bulbs to sub-optimal storage conditions to induce the aberrations.

The development of the disorder 'bud abortion' in tulip was studied by exposing the bulbs to long-term cold and dry storage. Bud abortion then manifests after planting in a short, unopened flower bud with white, papery tepals. Bud abortion was emerging after 23 weeks of storage and subsequent planting. When storage was continued, the shoot became fully aborted. The aberration resulted from the combined effects of storage and planting conditions. With the use of MRI, water concentration values and T_1 and T_2 relaxation times were determined non-invasively. T_1 and T_2 are indicative of the mobility of water molecules. Whereas T_1 is mainly influenced by the composition of the tissue sap, T_2 is also influenced by the anatomical structure of the tissue in question. The T_1 and T_2 relaxation times of the stamens, the *in vivo* determined water concentration of the shoot and the classically determined water content and osmolality of the scale and shoot tissue changed gradually throughout storage. These changes probably indicate a decreasing availability of water for root and shoot growth directly after planting, implying an increased risk of bud abortion. Planting at lower temperatures might enable the bulb to overcome its water deficiency by water uptake from the soil and enable the bulb to flower properly. This seems not longer possible in fully aborted bulbs. These bulbs do not seem to be able to initiate root or shoot elongation and water is probably withdrawn from the bud for growth of the daughter bulbs. This withdrawal of water from the shoot was first detectable in the stamens. The water content, water potential and *in vivo* water concentration in the stamens dramatically dropped. Degenerated parenchyma cells were observed in the stamens.

Apart from long-term cold storage, bud abortion in tulip can also be induced by storing bulbs at high temperatures. Application of a high-temperature treatment to abort the flower is used in practice to propagate the planting stock. To study whether the course of processes accompanying bud abortion is always similar, bud abortion was also induced by storage at high temperatures. A subsequent period of low temperatures was applied to fulfill the cold requirement of the bulbs. With increasing storage temperature and duration, flower quality shifted from normal flowers, to flowers with shriveled tepals and eventually to stems without a flower. The ion-leakage from the shoots of stored bulbs increased as well with the duration of the high-temperature pretreatment and seems therefore indicative of a decreased flower quality. After the cold requirement was fulfilled aberrations in the flowers could be visualized non-invasively with the use of T_2-weighted MRI. In contrast to long-term cold storage, storage at high temperatures did not affect the water status of the bulbs. Since the underlying processes appear to be different, more

specific definitions than the general term 'bud abortion' could and should be used to indicate the apparently different physiological disorders.

For the year-round production of lily flowers, bulbs are stored for long periods at subzero temperatures. The start of the freezing period and the freezing temperature should be chosen properly to minimize the risk of freezing injury. Because of the variability in optimal storage conditions between cultivars and years, freezing injury cannot be entirely prevented. The effects of freezing injury in lily bulbs were therefore assessed. Prolonged storage at too low temperatures resulted in freezing damage, manifesting with aberrant leaves and eventually aborted shoots. The decreased water potential and water content show that freezing damage involves dehydration of the damaged tissue. The decreased T_1 relaxation times in the stem and shoot apex of freezing damaged bulbs, determined non-invasively by MRI, also points towards desiccation of the tissue. Changes in the ion-leakage from the shoot during storage at too low temperatures, probably indicating changes in membrane permeability, coincided with the increased damage found after planting the bulbs. Changes in the ion-leakage of the scales and in the osmolality of the scales and shoot are probably indicative of an acclimation of the tissue to the applied storage temperatures.

Based on a preliminary study on severely damaged (water-soaked) lily bulbs it can be concluded additionally that water content, water potential and T_1 (and T_2) relaxation times can be used to distinguish different kinds of damage in lily bulbs.

The possibilities of MRI to assess non-invasively the internal quality of flower bulbs appear extensive. T_2-weighted imaging appeared to be a suitable application to visualize morphological changes like the growth of the shoot and the development of daughter bulbs. Aberrant developments of the floral parts in tulip bulbs after storage at high temperatures and the presence of aborted buds in *Hippeastrum* (amaryllis) bulbs could well be observed. Water concentration and T_1 and T_2 relaxation times were successfully used to assess the water status in the bulbs and to monitor water status related disorders like bud abortion in tulips after long-term cold and dry storage, freezing injury and water-soaking in lily bulbs.

Thus, MRI has a great potential for the evaluation of the internal quality of flower bulbs and horticultural products in general. However, currently the technique is still too expensive for routine, high-throughput applications, but on-line applications will become more feasible in the future.

Samenvatting

Veel bloembollen hebben een levenscyclus van één of meer jaren en bloeien in de lente of in de zomer. Toch is het jaarrond produceren van snijbloemen in de tuinbouw een normale zaak. De jaarrond produktie van bloemen vereist sturing van de bloeiperiode. De bollen krijgen daarom verschillende temperatuurbehandelingen om hun groei en ontwikkeling te beïnvloeden. Wanneer de bewaar- en plantomstandigheden niet optimaal zijn gekozen kunnen fysiologische afwijkingen ontstaan. Bloemknopverdroging en misvormde bladeren zijn voorbeelden van dergelijke fysiologische afwijkingen. Afwijkingen zijn meestal niet van buitenaf zichtbaar. Terwijl ze vaak al tijdens de bewaring zijn geïnduceerd, worden de afwijkingen pas zichtbaar na het planten.

Het doel van deze studie was meer inzicht te krijgen in de processen die van invloed zijn op het ontstaan van fysiologische afwijkingen in bloembollen, geïnduceerd door niet juiste bewaaromstandigheden. Er werd verwacht dat veranderingen in de waterstatus vaak betrokken zouden zijn bij de ontwikkeling van de geïnduceerde afwijkingen. De waterstatus van de bewaarde bloembollen werd daarom bestudeerd zowel met behulp van magnetische resonantie beeldvorming (of magnetic resonance imaging (MRI), bekend van medische toepassingen) als met klassieke bepalingen. Er is nagegaan of de gevonden parameters kunnen dienen als 'vroege indicatoren' voor een verminderde kwaliteit van een bloembol tijdens de bewaring en dus als indicatoren voor de interne kwaliteit van bollen. Dit biedt de mogelijkheid de aanwezigheid van afwijkingen op een vroeg stadium van ontwikkeling aan te tonen. Een dergelijke toets zou kunnen voorkomen dat bollen van een slechte kwaliteit worden bewaard of geplant en biedt de mogelijkheid de schade te beperken. Verder zou het optimalisatie van bewaaromstandigheden van nieuwe bloembolsoorten vergemakkelijken. Bovendien kan de bloemkwaliteit met behulp van een dergelijke toets beter worden gegarandeerd.

In deze studie is het mechanisme achter een aantal fysiologische afwijkingen in bloembollen bestudeerd door bollen bloot te stellen aan suboptimale bewaaromstandigheden die de afwijkingen induceren.

De ontwikkeling van de afwijking 'bloemknopverdroging' in tulpenbollen werd bestudeerd door de bollen langdurig onder koude, droge omstandigheden te bewaren. Bloemknopverdroging manifesteert zich dan na planten als een korte, ongeopende bloemknop met droge, witte tepalen. Knopverdroging werd zichtbaar nadat de bollen 23 weken waren bewaard en vervolgens geplant. Na langere bewaring aborteerde de spruit zelfs volledig. De afwijking bleek het gevolg te zijn van de gecombineerde effecten van de bewaar- en plant-omstandigheden. Met behulp van MRI werden waterconcentraties, T_1 en T_2 relaxatietijden niet-invasief bepaald. T_1 en T_2 zijn een aanwijzing voor de mobiliteit van watermoleculen. Terwijl T_1 voornamelijk wordt beïnvloed door de samenstelling van het weefselsap, wordt T_2 ook bepaald door de anatomische structuur van het weefsel in kwestie. De T_1 en T_2 relaxatietijden van de meeldraden, de *in vivo* bepaalde waterconcentratie van de spruit en de klassiek bepaalde watergehaltes en osmolaliteit van rok- en spruitweefsel veranderden geleidelijk gedurende de bewaring. Deze veranderingen zijn waarschijnlijk een indicatie voor de afnemende beschikbaarheid van water voor groei van de wortels en spruit direct na planten, hetgeen duidt op een toegenomen kans op knopverdroging. Planten bij lagere temperaturen geeft de bol de mogelijkheid zijn watertekort te boven te komen door wateropname vanuit de grond waardoor de bloem toch normaal kan bloeien. Dit lijkt niet meer mogelijk bij volledig geaborteerde bollen. Deze bollen kunnen geen wortel- en spruitstrekking initiëren. Het water is waarschijnlijk onttrokken aan de knop ten behoeve van de groei van de dochterbollen. De onttrekking van water aan de knop was allereerst zichtbaar in de meeldraden. Het watergehalte, de waterpotentiaal en de met behulp van MRI bepaalde waterconcentratie in de meeldraden daalden flink. In de meeldraden waren ook gedegenereerde parenchymatische cellen zichtbaar.

Bloemknopverdroging in tulpenbollen kan behalve door langdurige koude bewaring ook worden geïnduceerd door droge bewaring bij hoge temperaturen. Om te bestuderen of knopverdroging altijd volgens hetzelfde mechanisme verloopt, werd knopverdroging ook geïnduceerd door bewaring van bollen bij hoge temperaturen. Het gebruik van hoge temperaturen om de bloem te aborteren wordt in de praktijk toegepast voor het vermeerderen van het plantgoed. Een periode van lage temperaturen werd vervolgens toegediend om aan de koudebehoefte van de bollen te voldoen. Bij toenemende bewaar-temperatuur en -duur verschoof de bloemkwaliteit van normale bloemen naar

bloemen met verschrompelde tepalen en uiteindelijk naar stengels zonder bloem. De uitlek van ionen uit de bewaarde bollen nam eveneens toe met de duur van de bewaring bij de hoge temperaturen en lijkt daarom te duiden op een verminderde bloemkwaliteit. Nadat aan de koudebehoefte was voldaan, konden afwijkingen in bloemen niet-invasief zichtbaar worden gemaakt met behulp van T_2-gewogen MRI. In tegenstelling tot langdurige, koude bewaring had bewaring bij hoge temperaturen geen invloed op de waterstatus van de bollen. Omdat de onderliggende processen verschillend lijken, zouden meer specifieke definities dan de algemene term 'bloemknopverdroging' gebruikt kunnen en moeten worden om de blijkbaar verschillende fysiologische afwijkingen aan te duiden.

Voor de jaarrond produktie van leliebloemen worden bollen langdurig onder nul graden bewaard. De start van de vorstperiode en de vriestemperatuur moeten zorgvuldig worden gekozen om het risico van vorstschade te beperken. Vanwege de variatie in optimale bewaaromstandigheden tussen cultivars en tussen groeiseizoenen kan vorstschade niet geheel worden voorkomen. De effecten van vorstschade in leliebollen zijn daarom bestudeerd. Langdurige bewaring bij te lage temperaturen resulteerde in vorstschade hetgeen zich manifesteert als afwijkende bladeren en uiteindelijk in een geaborteerde spruit. De afgenomen waterpotentiaal en watergehaltes tonen aan dat vorstschade gepaard gaat met uitdroging van het beschadigde weefsel. De afgenomen T_1 relaxatietijden in de stengel en in het groeipunt van de spruit van de door vorst beschadigde bollen, bepaald met MRI, duiden ook op uitdroging van het weefsel. Veranderingen in de uitlek van ionen uit de spruit gedurende de bewaring bij te lage temperaturen, die waarschijnlijk een indicatie zijn voor veranderingen in de membraanpermeabiliteit, gingen gepaard met toename in schade, zoals gevonden na het planten van de bollen. Veranderingen in de uitlek van ionen uit de rokken en in de osmolaliteit van de rokken en de spruit zijn waarschijnlijk een aanwijzing voor aanpassing van de weefsels aan de bewaar-temperaturen.

Op basis van een voorlopige studie aan leliebollen, die onder water hebben gestaan, kan worden geconcludeerd dat watergehaltes, waterpotentialen en T_1 (en T_2) relaxatietijden kunnen worden gebruikt om verschillende soorten schade in leliebollen van elkaar te onderscheiden.

De mogelijkheden van MRI om niet-invasief de interne kwaliteit van bloembollen te toetsen zijn groot. T_2-gewogen beeldvorming is een goede methode om morfologische veranderingen in bollen, zoals de groei van de spruit en de ontwikkeling van dochterbollen, zichtbaar te maken. Afwijkende ontwikkelingen van de onderdelen van de bloem van tulpen na bewaring bij

hoge temperaturen en de aanwezigheid van verdroogde knoppen in *Hippeastrum* (amaryllis) bollen konden duidelijk worden aangetoond. Waterconcentraties en T_1 en T_2 relaxatietijden zijn goede parameters voor het karakteriseren van de waterstatus in bollen en de aan de waterstatus gerelateerde afwijkingen zoals bloemknopverdroging in tulpen na langdurige koude en droge bewaring en vorst- en waterschade in leliebollen.

De toetsing van de interne kwaliteit van bloembollen en tuinbouwprodukten in het algemeen met behulp van MRI biedt dus veel perspectief. Tot nu toe is het gebruik van de techniek echter te duur voor routinematige, grootschalige toepassingen, maar dergelijke on-line toepassingen zullen in de toekomst effectiever worden.

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Bedankt allemaal!

Curriculum vitae

Maaike van Kilsdonk werd geboren op 23 mei 1974 te Oss. In 1992 behaalde zij het Atheneum B diploma aan het Maaslandcollege te Oss. In dat zelfde jaar begon zij aan de studie Moleculaire Wetenschappen aan de toentertijd genaamde Landbouwuniversiteit Wageningen (LUW). Haar eerste afstudeervak stond onder begeleiding van Dr. J. Vervoort bij de vakgroep Biochemie aan de LUW en betrof het ophelderen van structuur-functie relaties van CF9 en AVR9 in het kader van het onderzoek naar de afweerreactie van de tomatenplant tegen de schimmel *Cladosporium fulvum*. Tijdens deze studie stond het gebruik van NMR centraal. Het tweede afstudeervak bij de vakgroep Plantenfysiologie (LUW) onder begeleiding van Ir. W. Wolkers was een studie naar de interacties tussen eiwitten en suikers in relatie tot droogte stress met behulp van FT-infrarood spectroscopie. Als laatste onderdeel van haar studie liep zij stage bij het Instituut voor Agrotechnologisch Onderzoek (ATO) gevestigd in Wageningen. In deze tijd bestudeerde zij het effect van 1-MCP op de ethyleengevoeligheid en houdbaarheid van *Cymbidium* orchideeën onder begeleiding van Dr. E. Woltering. In september 1997 behaalde zij het doctoraal diploma. In januari 1998 begon zij aan het in dit proefschrift beschreven promotie onderzoek bij de Universiteit Utrecht onder begeleiding van Prof. dr. C. Kollöffel en Prof. dr. K. Nicolay en gefinancierd door STW en NWO.