Long term storage of clonal material of lily (*Lilium* L.)

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Contents

Abbreviations

CGN	the Centre for Genetic Resources, The Netherlands
CPRO-DLO	DLO-Centre for Plant Breeding and Reproduction Research
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTPA	diethylene-triaminepenta-acetic acid
FTcon	freezing tolerance estimated by conductivity of external solution
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
IBPGR	the International Board for Plant Genetic Resources
IPGRI	the International Plant Genetic Resources Institute
LT50	the temperature that is lethal to 50 % of the tested material
TT50	the temperature at which 50 % of the scales had thawed
r_s	Spearman's rank correlation coefficient (Snedecor and Cochran, 1980)
SSA	sulfosalicylic acid
SH compounds	sulfhydryl compounds

Chapter 1

General introduction

Genetic variation in lily

Germplasm collections are important as source of genetic variation for breeding, research, and to prevent rare species from becoming extinct. The urgency to collect, describe, and store accessions of useful or potentially useful plant species leads to large efforts of genebanks and institutions to preserve germplasm collections. Therefore, it is important to preserve germplasm collections efficiently (Wehner, 1988). Several germplasm collections were defined by the International Board for Plant Genetic Resources (IBPGR) that later became the International Plant Genetic Resources Institute (IPGRI) (Guarino *et al.*, 1995). Base collections of germplasm are stored for secure long-term conservation. Accessions are only removed for regeneration or to provide material for an active collection. Material in active collections is continuously being removed for regeneration, multiplication, characterization, evaluation, or distribution. Working collections are active collections that also include special genetic stocks, such as breeders' lines and mutants.

The Centre for Genetic Resources, The Netherlands (CGN), bears international responsibility to maintain germplasm collections of *Allium*, *Brassica*, *Lactuca*, and *Solanum*. This genebank is incorporated in DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO). In addition, CPRO-DLO maintains several other germplasm collections to support breeding and research. One of the germplasm collections is a working collection of more than 1000 lily genotypes.

The lily (*Lilium* L.) is a perennial bulb, that is used worldwide as an ornamental. The centre of origin of the genus *Lilium* is the Himalayan Region (De Jong, 1974). The taxonomic classification divides the genus into seven sections (Comber, 1949; De Jong, 1974) with approximately 100 species in total. Species within three of the sections are the ancestors of

the three modern cultivar groups (Fig. 1.1, Van Tuyl *et al.*, 1996). The Asiatic hybrids present the first important group. They resulted from interspecific hybridization within the Sinomartagon section. For more than 30 years, the Asiatic hybrids were worldwide economically the most important group for cut flower production. Recently, the second group, the Oriental hybrids, became economically more important than the Asiatic hybrids. They were obtained by interspecific hybridization of species of the Archelirion section (McRae, 1976; Feldmaier and McRae, 1982). Most ancestors of the Asiatic and Oriental hybrids originate from China and Japan (Beattie and White, 1993). The third group comprises cultivars of the species *L. longiflorum* (section *Leucolirion*). They are also called Trumpet lilies or Easter lilies (Beattie and White, 1993). Until approximately 1980, the cultivation of *L. longiflorum* in the Netherlands was not possible, because of summer sprouting, and bulbs were imported from Japan. Through breeding activities, new cultivars have been obtained with a lower sensitivity to summer sprouting (Van Tuyl, 1985; Van Tuyl, 1992) and *L. longiflorum* bulbs are now exported from the Netherlands to Japan (Matsuo, 1992). In the Netherlands, the area of lily cultivation has increased from 227 ha in 1970 (Van Tuyl, 1992) to 3580 ha in 1995 (Anonymous, 1996).

The CPRO-DLO-lily collection contains a wide genetic variation for important traits, like flower colour and shape, low-light tolerance, forcing characteristics, flower longevity, and disease resistances. It has been used successfully to screen for new sources of resistance to *Fusarium oxysporum* (Straathof and Van Tuyl, 1994) and for flower longevity (Van der Meulen-Muisers *et a*l., 1995). Possibilities for combining the genetic potential of different species and groups (interspecific hybridization) have been increased by new pollination and fertilization techniques (Van Creij *et al.*, 1993; Van Tuyl, 1996; Van Tuyl *et al.*, 1991; Van Tuyl *et al.*, 1993; Van Tuyl *et al.*, 1996). Tetraploidisation techniques have restored fertility of sterile interspecific diploid hybrids (Van Tuyl, 1993). Cross pollination barriers between *L. longiflorum* and the Asiatic hybrids (LA hybrids) and recently between the Oriental and Asiatic hybrids (OA hybrids) have been overcome by application of these methods (Van Tuyl *et al.*, 1996).

Figure 1.1 (From Van Tuyl *et al.*, 1996) Crossing polygon of the genus *Lilium*. Large circles denote sections. The connection between the hybrid groups (ellipses) and their parents are shown by dotted lines. The solid lines denote successful cross combinations, with the arrows pointing towards the female parents.
Abbreviations: A: Aurelian hybrids; AL: *L. alexandrae*; AM: *L. amabile*; AS: Asiatic hybrids; AU: *L. auratum*; BU: *L. bulbiferum*; CA: *L. candidum*; CAN: *L. canadense*; CE: *L. cernuum*; CH: *L. chalcedonium*; CO: *L. concolor*; DAU: *L. dauricum*; DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum*; LA: *L. lankongense*; LEI: *L. leichtlinii*; LO: *L. longiflorum*; MA: *L. martagon*; MI: *L. michiganense*; MO: *L. monadelphum*; NO: *L. nobilissimum*; O: Oriental hybrids; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SP: *L. speciosum*; SU: *L. sulphureum*; TI: *L. tigrinum*; TS: *L. tsingtauense*; Test: *L. x testaceum*.

Preservation of lily germplasm

Lily bulbs for bulb production are usually planted in March-April and harvested in October-November. Subsequently, they are cooled at 2-5 □C for 6-10 weeks, depending on the cultivar, to break dormancy. Bulbs of many cultivars can be forced to flowering year-

round afterwards. If longer storage is required, the temperature must be lowered to -2 □C (Beattie and White, 1993; Van der Salm and Van der Salm, 1985). Lily bulbs exist of scale-like leaves attached to a compressed stem, the basal plate. These scales are used as storage organs. They can be used to propagate the lilies (Griffiths, 1933; Van Tuyl, 1983; Beattie and White, 1993). Scales can be detached, disinfected, and placed for eight weeks at 25 □C, followed by four weeks 17 □C, and 6-12 weeks 2-5 □C in vermiculite or in moist peat. Scale bulblets are formed adventively at the bottom of the scales. In the field or greenhouse, these scale bulblets can be grown to larger bulbs.

Genotypes of lily have to be preserved vegetatively as clones, because these genotypes are unique and heterozygous. Using seeds for preservation would break up unique genetic combinations. Collections of bulb crops are usually maintained in the field or greenhouse by yearly planting, harvesting and storing the bulbs, leading to high investments of labour and space and risking losses caused by diseases (Towill, 1988; Withers, 1991). Increasing the storage duration of the bulbs would make the maintenance of a field collection more efficient.

Conditions of minimal growth can be used to lengthen the storage duration of clonal material (Grout, 1991; Withers, 1991). Low temperatures are often used to create such conditions (Grout, 1991; Withers, 1991). Much research has been carried out to develop a suitable lily bulb storage method enabling year-round flower production. This has resulted in a method in which bulbs of Asiatic hybrids, Oriental hybrids and *L. longiflorum* are stored in moist peat at temperatures between -1.5 □C and -2 □C for 1 year. However, sometimes sprouts are damaged after bulb storage at -2 □C (Beattie and White, 1993; Boontjes, 1983). Increased freezing tolerance of lily would reduce the risk of freezing injury, and could make storage at a lower temperature than -2 □C possible. Freezing tolerance can be increased by cold-acclimatization, by abscisic acid, partial dehydration, or low atmospheric pressure (Halloy and Gonzales, 1993; Hincha, 1994; Lång *et a*l., 1994; Mantyla *et a*l., 1995). Storage at a lower temperature than -2 □C would further minimize growth conditions and would therefore increase maximum storage duration. In order to store a genetically diverse vegetative germplasm collection, knowledge about the variation in freezing tolerance among genotypes is needed. Furthermore, freezing tolerance has to be constant during storage, otherwise freezing injury could occur during prolonged storage.

The rate of metabolism of clonal material can also be decreased by enriching the atmosphere with CO_2 and decreasing the partial pressure of O_2. This modified atmosphere

can be achieved by controlled atmospheric storage (CAS) or by using modified atmosphere packaging in closed bags (MA package). An atmospheric equilibrium will develop in such bags, which contains a higher CO_2 concentration and a lower O_2 concentration. MA packages have been used to extend the storage life of many crops including pre-cooled tulip bulbs (Prince *et a*l., 1981; Prince *et a*l., 1986). MA packages have several advantages over CAS. Firstly, material is divided into small batches, reducing the risk of quickly spreading diseases. Secondly, the use of MA packages is much cheaper than the application of technical equipment for CAS. However, a disadvantage of MA packages is the less accurately controlled atmosphere.

An alternative method to preserve a lily collection, is conservation *in vitro*. *In vitro* stored collections need relatively small amounts of space, medium components can be used to minimize growth, plants can be multiplicated quickly, and there is often a possibility to eliminate viral diseases (Towill, 1988; Withers, 1991). However, at each transfer event, there is a risk of contamination with microbial organisms (Withers, 1991). Furthermore, the establishment of an *in vitro* collection is labour-intensive and genotypes may react differently under identical conditions (Towill, 1988).

In the future, cryopreservation of lily meristems could be a suitable method for long-term preservation. Research by Bouman and de Klerk (1990) resulted in the survival of 8 percent of meristems of *L. speciosum* 'Rubrum'. Apical meristems from scale bulblets of *L. japonicum* Thunb. have been successfully cryopreserved by using the technique of vitrification. The rate of shoot formation after cryopreservation was approximately 80 % after 4 weeks. Later this vitrification method was also successfully applied to five other lily genotypes (Matsumoto *et al.*, 1995).

Viability loss of bulbs

The physiological and biochemical processes causing viability loss during storage are rather unknown for lily bulbs. Insight into these processes would facilitate the development of better storage techniques.

Formation of scale bulblets on scales is dependent on carbohydrate reserves during storage. Bulbs lose part of their carbohydrate reserves during storage by respiration, but bulbs contain large amounts of carbohydrates. For *L. longiflorum* 'Nellie White', bulb scales

contained approximately 700 mg starch per gramme dry matter (Miller, 1990). Moreover, lily bulbs have low respiration rates compared to, for instance, apples (De Hertogh and Le Nard, 1993). Therefore, loss of carbohydrates is not likely to be a limiting factor during storage of lily bulbs under conditions of minimal growth.

Partial dehydration of bulbs can also lead to viability loss, but protecting against dehydration is easy by using moist peat and/or a package that diminishes evaporation. *Penicillium* or other infesting microbial organisms can also cause loss of viability (De Hertogh and Le Nard, 1993). Therefore, bulbs should also be disinfected before storage.

Free radical-induced oxidative stress is thought to be a major process in the loss of viability during ageing of plant tissues (Benson, 1990; Kumar and Knowles, 1993; Leshem, 1988; McKersie *et al.*, 1988; Paliyath and Droillard, 1992; Van Bilsen *et al.*, 1994b). Oxidative stress is also involved in loss of viability of plants exposed to a variety of environmental stress situations, such as chilling (Hariyadi and Parkin, 1991), freezing (Steponkus, 1984), and pathogenic diseases (Bradley *et al.*, 1992). Oxidative stress was expected to limit storage duration of bulbs.

Oxidative stress leads to breakdown of membrane phospholipids, and therefore, increases membrane permeability (Benson, 1990; Leshem, 1988; McKersie *et a*l., 1988). Free radicals can de-esterify phospholipids in a membrane bilayer (McKersie *et al.*, 1988). De-esterification of phospholipids leads to a decrease in the content of phospholipids and an increase in the content of free fatty acids. Attack on double bonds of unsaturated fatty acids by free radicals can lead to chain reactions, causing further breakdown of unsaturated fatty acids, increasing the level of saturation of the remaining fatty acids (Benson, 1990). Chain reactions can also lead to polymerisation of cell wall structural proteins (Bradley *et a*l., 1992). Further peroxidation of fatty acids leads to small organic compounds as ethane, jasmonic acid, malondialdehyde and pentane.

Numerous defence mechanisms are active in higher plants, protecting cells from damage by free radicals (Benson, 1990; Leshem, 1988). Free radical scavengers, like superoxide dismutase, ascorbic acid (vitamin C), and I-tocopherol (vitamin E) decrease the population of free radicals in tissues by reacting with free radicals already present. Peroxidative enzymes can prevent the formation of free radicals by enzymatical breakdown of peroxide groups. Cytokinins can react both as free radical scavengers and as inhibitors of free radical formation. Glutathione (GSH), the reduced form of the tripeptide, L-I-glutamyl,-L-

cysteinyl-glycine, protects tissue components from oxidative breakdown by providing a preferential substrate for oxidation. GSH also functions in reducing peroxide groups and hence free radical production (Benson, 1990; Leshem, 1988). Decline in GSH content and increase of the oxidative state of glutathione (GSSG / GSH) indicate a less effective defence against oxidative stress (De Vos *et a*l., 1994).

Other causes of viability loss during storage could be phase separation of membrane phospholipids and freezing injury, both resulting in increased membrane permeability. Phase separation of membrane phospholipids can result from phase transition. The phase transition temperature is dependent on the water content of the tissue and the composition of the fatty acids in the phospholipids (Hoekstra, 1989), which can change during storage.

Outline of the thesis

Eliminating one or more seasons of bulb cultivation by long term bulb storage would reduce the costs for preserving lily collections. Therefore, research was carried out to develop techniques for long term storage of lily bulbs. Knowledge about the physiological and biochemical processes that limit maximum storage duration, would facilitate the development of better storage techniques (Chapter 1).

Techniques for measuring the viability are necessary to detect decline of vigour during storage. The formation of scale bulblets on detached lily scales is needed for the propagation of the lily bulb and can therefore be used to asses the viability of lily bulbs. In addition, ion leakage from scales might be a usable criterion for viability of lily bulbs. Research on ion leakage is described in Chapter 2 (damage by freezing, heat, or drying out) and Chapter 3 (viability loss during storage).

Techniques for long term storage of clonal material of lily can be developed by storing clonal material under various conditions and measuring viability regularly. Viability loss of lily genotypes during storage of bulbs in moist peat at -2 □C has been studied in Chapter 4.

The use of polyethylene bags for storing lily germplasm is described in Chapter 5. With these bags an internal atmosphere can be achieved with an increased CO_2 and a decreased O_2 concentration, and the lily germplasm can be prevented from dehydration. Viability loss of scale bulblets of various lily genotypes has been determined during long-term storage in polyethylene bags at different temperatures.

Chapter 6 describes viability loss during *in vitro* storage of several lily genotypes under growth retarding conditions. Low temperature, a dormancy-inducing temperature, low minerals, and high sucrose contents have been studied for their effects on growth and viability.

Effects of genotype, previous bulb storage duration, freezing exposure, and partial dehydration of lily bulb scales have been studied in relation to freezing tolerance of lily bulb scales (Chapter 7).

The role of oxidative stress as a process leading to loss of viability of lily bulb scales stored at -2 □C in moist peat has been studied by measuring changes in ion leakage, the content and oxidation state of glutathione, the content of phospholipids, the content of neutral lipids, the content of free fatty acids, and the degree of unsaturation of fatty acids in phospholipids during storage (Chapter 8).

In the general discussion (Chapter 9), the prospects of the results for application in germplasm preservation are discussed.

Chapter 2

Conductivity and potassium leakage as indicators for viability of vegetative material of lily, onion and tulip

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Abstract

The preservation of a broad genetic variation in collections of germplasm is important for breeding crops and combatting genetic erosion. To maintain germplasm collections of bulb crops, research is started to develop methods for long term storage of vegetative material. To develop these methods, techniques for measuring the viability are necessary. Experiments aiming to find an early indicator for viability of vegetative material of lily, onion and tulip were executed. The viability of the material was decreased artificially by frost, heat or drying out treatments. After this treatments, scales of lily and bulbs of onion and tulip were cleaned and placed in distilled water for one and a half hour. Afterwards, conductivity and potassium leakage were measured and the material was planted to observe growth and development. In all instances of severe damage, death of the material was accompanied by high values of conductivity and potassium leakage.

Introduction

The preservation of a broad genetic variation in collections of germplasm is important for breeding crops and combatting genetic erosion. To maintain germplasm collections of lily and tulip, in 1991 at CPRO-DLO research was started to study techniques for long term storage of clonal material of these crops. In Gatersleben, a project was started with the aim to develop methods for long term storage of vegetative material of *Allium*. To study preservation of biological material, techniques for measuring the viability are necessary.

During ageing, several processes lead to the destruction of membranes in plant cells. Oxidation induced by free radicals, leads to increased membrane permeability and a higher level of leakage of nutrients from cells (Benson, 1990; Leshem, 1988). Membrane leakage can also be caused by phase separation of membrane phospholipids, which appears at a certain temperature depending on the water content of the tissue (Hoekstra, 1989). Freezing injury results in increased membrane permeability as well (Grout, 1991; Withers, 1991).

The described processes leading to membrane leakage suggest this effect can be used to measure loss of viability. For determining the quality of seeds, ion leakage from cells measured by conductivity is used as a standard test (Anonymous, 1983; Schmidt and Tracy, 1988; Tracy and Juvik, 1988). Ion leakage measured by conductivity and potassium leakage also appeared to be a good instrument to measure damage of ozone exposure in *Phaseolus vulgaris* (McKersie *et al.*, 1981). Forney and Peterson (1990) reported that enhanced leakage of K ions was an effective indicator of chilling injury in grapefruit callus. However McCollum and McDonald (1991) found that electrolyte leakage from chilled fruit measured by conductivity did not increase significantly until chilling injury had become severe. The aim of our research was to test the relationship between ion leakage and decrease of viability in vegetative material of the the bulb crops lily, onion and tulip. To compare vegetative material with different viability, the viability of part of the material was decreased artificially by frost, heat or drying out. Leakage was determined by measuring both conductivity and potassium leakage.

Material and methods

Plant material

Onion: fresh yielded bulbs of 'Stuttgarter Zefa'.

Lily : Bulbs of 'Milano', 'Connecticut King', 'Star Gazer' and *L. longiflorum* 'Gelria' stored for at least one year in moist peat at -2 □C.

Tulip: Bulbs of 'Apeldoorn' and 'Lustige Witwe' stored dry at 20 □C until about two months after stage G (floral development completed).

Leakage tests

From bulbs of onion and tulip, the outer scales were removed until the first scale with no visible damage. From lily bulbs the five most healthy scales were selected from scales at the inner part of the bulbs. Then the material was washed and stored for one day at room temperature to give fresh wounds time to heal. After that day the material was washed in distilled water and put for 1.5 hour in 100 ml distilled water to leak nutrients. Afterwards the conductivity of the distilled water was measured using a conductivity meter. The potassium concentration was determined using a flame photometer.

Survival assessments

After the leakage test, the survival percentages of the material used were scored as follows: from bulbs of onion the upper parts of the whole bulbs were cut off and removed. The appearance of developing sprouts after planting in wet vermiculite was scored. Separate lily scales were planted in wet vermiculite and the appearance of scale bulblets was scored after eight weeks of incubation at 25 □C. Whole bulbs of tulip were forced in a greenhouse after a cold period (2 □C) of 16 weeks. The appearance of developing sprouts was scored.

Experiments

Three experiments were done to induce reduced viability.

Experiment 1: severe cold and heat

Five bulbs per treatment of onion 'Stuttgarter Zefa', lily 'Milano' and tulip 'Apeldoorn' were stored in open polyethylene bags for three days at the temperatures -20 □C, +50 □C

and the control temperatures -2 □C and +2 □C. After storage ion leakage and survival percentages were determined.

Experiment 2: increasing temperature

Five bulbs of onion 'Stuttgarter Zefa', lily 'Connecticut King' and tulip 'Lustige Witwe' were placed at 2 □C and 25 bulbs in a stove at 38 □C. Each day a random sample of five bulbs of each genotype was taken out of the stove and placed together with the control bulbs at 2 □C. The bulbs were stored in polyethylene bags to prevent them from drying out. Each time bulbs were taken out, the temperature was increased with some degrees. During the period of four days, the temperature in the stove was increased from 38 □C via 42 □C and 45 □C to 50 □C. After one week ion leakage and survival percentage tests were done.

Experiment 3: drying out

Bulbs of lily 'Connecticut King', 'Star Gazer' and 'Gelria' were stored dry at the temperatures -2 □C, +2 □C and +35 □C. From five bulbs of each cultivar ion leakage and survival percentages were tested immediately. After one, three and eight weeks, a random sample of five bulbs per treatment per genotype was taken. Ion leakage and survival percentages were scored.

Results

In all experiments, a high correlation between conductivity and potassium leakage was found. Varying from 0.976 (exp. 3) to 0.989 (exp. 2).

In the first experiment, a relative high ion leakage was determined for all genotypes after storage at -20 □C or +50 □C and none of the material survived that treatment (Table 2.1). Differences between the results at -2 □C and +2 □C were not significant.

Table 2.1 Mean conductivities (Con.) ($TS.cm^{-1}$), potassium leakages (K-leak) ($mg.l^{-1}$) and survival percentages (Surv.) of bulbs of lily 'Milano', onion 'Stuttgarter Zefa' and tulip 'Apeldoorn', stored for three days at different temperatures.

Genotype	Test	Temperature (°C)			
		-20	-2	+2	+50
Lily 'Milano'	Con.	128.00	0.88	0.80	80.60
	K-leak	28.06	0.11	0.16	18.66
	Surv.	0	100	100	0
Onion 'S.Zefa'	Con.	72.84	7.74	5.40	29.88
	K-leak	19.20	1.56	0.76	7.86
	Surv.	0	100	100	0
Tulip 'A.Doorn'	Con.	28.88	1.56	1.46	23.88
	K-leak	8.84	0.29	0.26	6.94
	Surv.	0	100	100	0

In the second experiment (Table 2.2), an increase of the temperature until and including 42 °C did not result in a significant increase of ion leakage for all genotypes used, except for 'Connecticut King', for which the determined conductivity at 2 °C was little but significant lower than at higher temperatures. This effect was not found for potassium leakage. With 'Connecticut King' both conductivity and potassium leakage were significantly higher at 45 °C and 50 °C than at all lower temperatures. High leakage after storage at 50 °C was accompanied by a poor survival percentage. At 45 °C however, all scales survived the treatment. For 'Stuttgarter Zefa' only at 50 °C leakage was significantly higher than at 2 °C. Though all bulbs survived the treatments, sprouting after storage at 50 °C was relatively poor compared with lower temperatures. Results with 'Lustige Witwe' were similar with 'Stuttgarter Zefa', although none of the 'Lustige Witwe' bulbs survived storage at 50 °C.

Table 2.2 Mean conductivities (Con.) ($TS.cm^{-1}$), potassium leakages (K-leak) ($mg.l^{-1}$) and survival percentages (Surv.) of bulbs of lily 'Milano', onion 'Stuttgarter Zefa' and tulip 'Lustige Witwe', stored at 2 □C and 38 □C with an daily increased temperature. The letters in the table give the horizontal significances ($P < 0.05$).

Genotype	Test	Temperature (□C)									
		2		38		42		45		50	
Lily 'C.King'	Con.	0.50	a	0.70	b	0.78	b	1.05	c	1.90	d
	K-leak	0.08	a	0.08	a	0.07	a	0.17	b	0.63	c
	Surv.	100		100		100		100		16	
Onion 'S.Zefa'	Con.	2.76	a	3.76	ab	2.10	a	3.33	ab	4.94	b
	K-leak	0.56	ab	0.66	b	0.35	a	0.66	b	1.26	c
	Surv.	100		100		100		100		100	
Tulip 'L.Wit.'	Con.	4.70	a	4.05	a	5.14	a	5.92	a	44.60	b
	K-leak	0.99	ab	0.64	a	0.87	ab	1.42	b	11.21	c
	Surv.	100		100		100		100		0	

In experiment 3 the viability was reduced by drying out. Of this experiment, no data are presented. At the control temperatures -2 □C and +2 □C, only small or no increases in ion leakage was observed and all material survived the eight weeks of storage. At 35 □C, bulbs of the three lily cultivars were dried out after eight weeks, in such a way that all the scales of these bulbs were dead and high levels of ion leakage were determined. With 'Connecticut King' leakage was only significant higher after eight weeks storage at 35 □C. Significant increase in ion leakage was found for both 'Gelria' and 'Star Gazer' after three weeks 35 □C. The increase in ion leakage was related to a decrease of the survival

percentages. Especially 'Star Gazer' was sensitive for drying out. After three weeks 35 □C all scales of the 'Star Gazer' bulbs were dead.

Discussion

In all experiments, damaged tissue showed high ion leakage indicating that ion leakage is a general process following injury of biological material. In some instances, ion leakage was increased significantly without visible damage, indicating ion leakage can be used as an early indicator for injury. However, it is not clear that the artificially induced damage in the experiments is representative for reduced viability after long term storage under optimal conditions. Also other causes of damage, like starvation and infection to pathogens may be important.

More knowledge is needed about the relationship between ion leakage and decrease of viability. The level of ion leakage probably varies with different kinds of damage. Moreover, probably only ion leakage from epidermal cell layers is measured. It is possible that in some instances high levels of ion leakage are caused by dead tissue that is not important for the regeneration of the material. Ion leakage might not be a criterion for viability that is sensitive enough to detect small decreases of viability (McCollum and McDonald, 1991).

In the second experiment the temperature of 50 □C was not as destructive as in the first experiment. This is probably due to a shorter storage duration and the acclimatization of the material in the second experiment in comparison to that in the first experiment.

The correlation between conductivity and potassium leakage was high in all experiments. Significances using conductivity or potassium leakage were almost equal. These results indicate that both conductivity and potassium leakage are similar in measuring nutrient leakage. As the measurement of conductivity is quicker than that of potassium leakage, in future experiments conductivity will be used.

Further research will concentrate on a quick and early method to measure viability. Relations between ion leakage and decrease of viability during storage will be studied in more detail.

Chapter 3

Ion leakage as a criterion for viability of lily bulb scales after storage at -2 □C for 0.5, 1.5 and 2.5 years

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Abstract

The viability of lily (*Lilium* L.) bulb scales of 'Avignon', 'Esther' (Asiatic hybrids), 'Star Gazer' (Oriental hybrid), and 'Snow Queen' (*L. longiflorum* Thunb.) was determined after storage at -2 □C for 0.5, 1.5 and 2.5 years. Ion leakage, the percentage of scales that formed bulblets and the number and weight of these bulblets were determined on scales from the inner, middle and outer part of bulbs. During storage, the outer scales of all cultivars and the inner and middle scales of 'Snow Queen' showed increased ion leakage accompanied by a decreased ability to form scale bulblets during storage. Concurrently, the percentage of scales forming bulblets declined and more and smaller scale bulblets were formed per regenerative scale. Thus, ion leakage is a useful criterion to measure viability of lily scales.

Introduction

The preservation of a broad genetic variation in germplasm collections is important for breeding crops and combatting genetic erosion (Grout, 1991; Wehner, 1988). Collections of clonal lines are usually maintained in the field (Towill, 1988), where there are high labour and space costs and risk of disease losses. Hence, storage of clonal material for several years would be very useful. A project was started at CPRO-DLO in 1991 with the goal of developing storage methods for lilies.

Techniques for measuring the viability are necessary to detect decline of vigour under diverse circumstances. In addition to growth characteristics, ion leakage might be a usable criterion, which is used as a standard test for determining seed viability (Schmidt and Tracy, 1988; Tracy and Juvik, 1988). Ion leakage measured by conductivity and potassium leakage can also be used to measure damage of ozone exposure in bean (*Phaseolus vulgaris* L.) (McKersie *et al.*, 1981). Forney and Peterson (1990) reported that enhanced leakage of K^+-ions was an effective indicator of chilling injury in grapefruit (*Citrus paradisi* Macf.) callus. However, McCollum and McDonald (1991) found that electrolyte leakage from chilled fruit measured by conductivity did not increase significantly until chilling injury had become severe. Electrolyte leakage has also been shown to be a good indicator for temperature stress in leaf disks of red pepper (*Capsicum annuum* L.) (Nanaiah and Andersen, 1992). Bonnier *et al.* (1992) showed that ion leakage measured by conductivity or potassium leakage can be used to determine severe injury in lily scales artificially damaged by frost, heat or desiccation.

To retard flowering, Asiatic and Oriental lily bulbs are usually stored at -2 □C (Beattie and White, 1993). *L. longiflorum* can also tolerate this temperature (Miller, 1993). The objective of our study was to test whether ion leakage of bulb scales is a satisfactory criterion for testing viability of lily bulbs stored at -2 □C. Therefore effects of storage duration were tested on viability of lily scales measured by ion leakage and formation of scale bulblets.

Materials and Methods

Plant material

'Avignon' and 'Esther' (Asiatic hybrids), 'Snow Queen' (*L. longiflorum* Thunb.) and 'Star Gazer' (Oriental hybrid) bulbs were obtained from commercial stocks. Of each cultivar, 300 bulbs with circumference of 14 to 16 cm harvested in October or November 1989, 1990 and 1991, were packed in moist peat and acclimatized for 6 weeks at 0 □C and then stored at -2 □C for 2.5, 1.5 and 0.5 years respectively.

In May 1992, 12 bulbs were sampled per cultivar and year of harvest. One scale from the inner 20 % of the scales (inner scale), one scale not belonging to the 40 % inner or outer scales (middle scale), and one scale from the outer 20 % of the scales (outer scale) was selected per bulb. The weight, ion leakage, and the formation of scale bulblets were measured for each scale.

Ion leakage

Scales were washed with water and stored during the night in a polyethylene bag at 18-20 □C to give fresh wounds time to heal. The next day each scale was washed in distilled water and then put for 1.5 h in 100 ml distilled water at 20 □C. Subsequently, the conductivity of the samples was measured using a digital conductivity meter (Philips PW9526 with electrode PW9514/60). The conductivity of each scale was corrected by subtracting the conductivity of the distilled water (1.85 $TS.cm^{-1}$) and then divided by the fresh weight of the scale.

Formation of scale bulblets

Scale bulblet formation was recorded after 8 weeks of incubation in vermiculite saturated with water at 25 □C. This method induces bulblet formation for Asiatic hybrids and *L. longiflorum* (Van Tuyl, 1983) and Oriental hybrids (Hendriks, 1988). The number and weight of the formed bulblets were determined per scale.

Statistical methods

Effects of storage duration on ion leakage and the formation of scale bulblets were analyzed by standard analysis of variance (ANOVA) and LSD. To get a normal distribution,

conductivity values were log-transformed. The mean conductivity values expressed in Table 3.1 are back transformed mean logarithm values. The number, weight and size of the scale bulblets formed per scale were analyzed, excluding scales that did not form bulblets. First, the model (cultivar * scale * -2 □C storage duration) was analyzed; but, for all characteristics a significant interaction was found between effects of storage time and of scale position. Therefore, statistical analyses were carried out separately for the inner, middle and outer scales. In absence of interaction between effects of cultivar and storage time, significances are displayed (Table 3.1) for main effects of storage time per scale type. Otherwise, significance is also presented per cultivar. The percentage of scales forming bulblets was not statistically analyzed per single scale because of extreme data (0 and 100%). The percentages were calculated per treatment.

To analyze the relation between conductivity and the formation of scale bulblets, the scales were classified into four categories of conductivity. Regression analysis was carried out with the number of bulblets formed per gram per regenerative scale, fresh weight of the scale bulblets formed per gram per regenerative scale and the mean fresh weight per scale bulblet using the model (category * cultivar * scale). Since all interactions were either not significant or small compared to main effects, means and a 95 % confidence interval were calculated over all scales per category.

Results

Effects of storage

Ion leakage, expressed as conductivity, increased significantly with storage duration for outer scales of all cultivars (Table 3.1). For 'Snow Queen', ion leakage of inner and middle scales increased also significantly with storage duration (Table 3.1). For the other cultivars, there was no significant effect of storage duration on ion leakage of inner and middle scales.

The regeneration % for inner and middle scales was more than 80 % in all treatments (Table 3.1). For outer scales the regeneration % decreased with storage duration depending on the cultivar. For outer scales of 'Esther' it was still 83 % after a storage duration of 2.5 years, but for 'Snow Queen' it was curtailed to 18 %.

The effect of storage time on the number of the scale bulblets varied per cultivar (Table 3.1). With 'Snow Queen', an increase in the number of scale bulblets was found on scales stored for 2.5 years for all scale types, although not significant for the outer

Table 3.1 Mean values of conductivity and the formation of scale bulblets for twelve inner, middle and outer scales after storage of bulbs of four lily cultivars at -2 □C.

Characteristic	Storage duration (years)	Inner scales					Middle scales					Outer scales				
		Cultivar					Cultivar					Cultivar				
		AV[z]	ES	SQ	SG	Mean	AV	ES	SQ	SG	Mean	AV	ES	SQ	SG	Mean
Conductivity	0.5	0.76 b[y]	0.63 a	1.00 a	0.65 a	0.75 a	0.71 b	0.45 ab	0.52 a	0.57 a	0.55 a	0.42 a	0.48 a	1.17 a	0.37 a	0.54 a
($TS.cm^{-1}.g^{-1}$)	1.5	0.42 a	0.59 a	1.80 b	0.63 a	0.73 a	0.34 a	0.26 a	0.90 b	0.69 a	0.49 a	0.65 a	0.70 ab	2.57 b	1.55 b	1.16 b
	2.5	0.65 ab	1.03 a	5.12 c	0.67 a	1.23 b	0.61 b	0.61 b	4.13 c	0.65 a	1.00 b	1.94 b	1.36 b	23.59 c	2.43 b	3.50 c
Regeneration %	0.5	100	100	100	92	98	100	100	100	100	100	100	100	100	92	98
	1.5	100	92	92	92	94	100	100	92	83	94	100	100	83	92	94
	2.5	100	100	83	92	94	100	100	83	92	94	50	83	18	50	51
Scale bulblets	0.5	3.3 b	2.9 a	2.8 a	0.9 a	2.5 a	3.6 b	2.0 b	1.6 a	0.6 a	2.0 b	2.5	1.8	2.6	0.9	2.0 a
per gram scale	1.5	2.1 a	2.7 a	2.5 a	1.2 a	2.1 a	2.2 a	1.1 a	1.5 a	1.2 a	1.5 a	2.9	2.7	2.6	1.8	2.5 b
(no.)	2.5	2.1 a	2.2 a	4.7 b	1.3 a	2.5 a	1.8 a	1.9 b	3.2 b	0.8 a	1.9 b	3.0	1.5	4.0	1.3	2.0 ab
Mean weight	0.5	0.06	0.13	0.08	0.09	0.09 a	0.09 a	0.24 a	0.14 a	0.23 a	0.17 a	0.20 a	0.21 a	0.24 a	0.47 b	0.28 a
per scale	1.5	0.16	0.12	0.09	0.18	0.14 b	0.29 b	0.48 b	0.17 a	0.40 b	0.33 b	0.28 a	0.26 ab	0.24 a	0.26 a	0.26 a
bulblet (g)	2.5	0.19	0.18	0.08	0.16	0.16 b	0.40 b	0.30 a	0.12 a	0.40 b	0.31 b	0.18 a	0.36 b	0.18 a	0.32 a	0.30 a

[z] AV = 'Avignon'; ES = 'Esther'; SQ = 'Snow Queen'; SG = 'Star Gazer'

[y] Mean separation between different storage durations by protected LSD test, P=0.05

scales. Significant differences in the number of scale bulblets of the other cultivars appeared to be effects of differences between samples grown in different seasons rather, than effects of storage time. Differences in the number of scale bulblets formed per gram between scale types were small.

The weight of the scale bulblets formed on inner scales increased significantly with storage time (Table 3.1). For 'Avignon' and 'Star Gazer', the same effect was found for middle scales. For outer scales, significant effects of storage duration on the weight of the scale bulblets were only found for 'Esther' and 'Star Gazer'. These significant differences are probably caused by sample differences. For all cultivars, the weight of the scale bulblets formed were larger on middle and outer scales than on inner scales (Table 3.1).

The relation between conductivity and the formation of scale bulblets

For most of the scales, the conductivity value was lower than 1 $TS.cm^{-1}.g^{-1}$ (conductivity class 0-1 in Table 3.2). With the exception of two scales, all the scales in this class regenerated scale bulblets. With increasing conductivity values, the number of regenerative scales declined relatively and the regenerative scales appeared to form more, but smaller scale bulblets (Table 3.2).

Table 3.2 Relation between ion leakage and formation of scale bulblets of lily scales, combined for four cultivars stored at -2 □C for 0.5, 1.5 or 2.5 years, classified in four categories of conductivity ($TS.cm^{-1}.g^{-1}$).

Conduc-tivity class	Scales (no.)	Regenerative scales (%)	Bulblets per gram scale (no.)	Mean weight per scale bulblet (g)
0- 1	279	99	1.9 ± 0.1^{z}	0.30 ± 0.02
1- 3	86	91	2.4 ± 0.3	0.29 ± 0.03
3- 9	40	68	3.1 ± 0.5	0.14 ± 0.06
> 9	22	27	5.4 ± 1.2	0.10 ± 0.15

[z]95 % confidence intervals

Discussion

Effects of storage

Ion leakage of outer scales increased with storage duration and it was accompanied by a decrease in the ability of scales to form new bulblets. With inner and middle scales, the increase in ion leakage and the loss of the regeneration % was limited to 'Snow Queen'. For all cultivars, effects of storage duration were most significant with outer scales. The faster decline of viability of outer scales compared to inner and middle scales was also found by Matsuo and Arisumi (1978) after storage of *L. longiflorum* bulbs at 25 □C. There may be several causes for this effect. First, outer scales are formed prior to the middle and inner scales and are therefore older. Second, outer scales are more exposed to environmental conditions and microorganisms.

For inner scales of all cultivars and for middle scales of 'Avignon' and 'Star Gazer', the weight of the bulblets formed increased with storage duration (Table 3.1). This increase in the weight of the bulblets was not accompanied by a decrease in the number of bulblets formed, except for 'Avignon'. The increase in the production of scale bulblets with storage duration has not been reported before.

Relation between conductivity and the formation of scale bulblets

Since the percent of regenerative scales declined with conductivity (Table 3.2), conductivity values can be used to predict regeneration of scales and to measure the effects of treatments on the viability of scales. However, ion leakage is not an absolute criterion to predict regeneration of scales. Some scales with high conductivity values still produced scale bulblets and two scales with conductivity < 1 $TS.cm^{-1}.g^{-1}$ did not (Table 3.2).

The observation that scales with high conductivity values formed more and smaller scale bulblets supports the results of Matsuo and Arisumi (1978). They found the same tendency for scales of *L. longiflorum* after treatment with hot water. It might be a general feature of lily scales with lowered viability.

Conductivity values are often presentated as a percentage of a maximum conductivity based on leakage after freezing or heating (Forney and Peterson, 1990). In this study conductivity values are not corrected for maximum conductivity values for two reasons. First, maximum conductivity values of the scales could not be established, as the scales were used

for propagation after measuring conductivity. Second, ion leakage of healthy scales was very low and probably limited to the outer cell layers of the scales. In that case, correction for maximum conductivity values of these scales would not be valid. Conductivity values that varied between 123 (ln(123) = 4.81) and 272 (ln(272) = 5.61) $TS.cm^{-1}$ per gram per scale were found after autoclaving and grinding of other lily scales (results not shown). Since conductivity values were distributed normally after a transformation to their logarithms, the distinction between their logarithms is more relevant for the variation in maximum conductivity than the distinction between the untransformed values.

In this study, ion leakage measured by conductivity appeared to be a useful criterion to measure viability of lily scales. It can be used to further investigate the development of suitable storage methods for lilies.

Chapter 4

Long term storage of lily bulbs at -2 □C

Bonnier, F.J.M.[1], A. Tribulato[2], and J.M. van Tuyl[1], 1997. In preparation.

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Abstract

For an efficient maintenance of a lily collection, the maximum storage duration of *Lilium* bulbs stored at -2 □C in moist peat was determined. 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Asiatic hybrids), 'Star Gazer' (Oriental hybrid), 'Gelria', and 'Snow Queen' (*L. longiflorum*) were used. The viability was determined by the percentage of bulbs with at least one regenerative scale (bulb regeneration), the proportion of regenerative scales (scale regeneration), and ion leakage from inner scales. Maximum storage duration based on bulb and scale regeneration varied between 2.9 and 4.0 years for the Asiatic hybrids and between 2.0 and 2.4 years for the other cultivars. Ion leakage of inner scales was increased for all cultivars at a storage duration of 3 years except for 'Enchantment' and 'Mont Blanc'. It was concluded that a lily collection can probably be effectively stored for 2 years at -2 □C in moist peat.

Introduction

Crop improvement depends on available genetic diversity. Therefore, breeding companies and research institutes must preserve germplasm collections. Lilies must be preserved vegetatively as clones, because using seeds would affect the unique genetic combinations. Collections of bulb crops are usually maintained by yearly planting, harvesting, and storing of the bulbs. This requires high investments of labour and space and the risk of losses caused by diseases (Towill, 1988; Withers, 1991). Eliminating one or more seasons of bulb growing by long term bulb storage would reduce costs for maintaining a lily collection. Therefore, research has been started to develop techniques for long term storage of lily bulbs.

Bulbs of Asiatic hybrids, Oriental hybrids and *L. longiflorum* are stored in moist peat at -2 □C for year-round forcing of lily bulbs (Boontjes, 1983; Beattie and White, 1993). The objective of the present study was to determine the maximum storage duration of lily bulbs stored under these conditions. Lily bulbs can be propagated by regeneration of scale bulblets from detached scales (Van Tuyl, 1983). The ability of scales to form scale bulblets and ion leakage from inner scales (Bonnier *et al.*, 1994) were used to measure bulb viability.

Material and methods

Plant material

Over a five year period, 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Asiatic hybrids), 'Star Gazer' (Oriental hybrid), 'Gelria', and 'Snow Queen' (*L. longiflorum*) bulbs (12/16 cm, in circumference) were obtained from commercial growers. The bulbs were harvested in October/November, dipped in the fungicide 1% captan and 0.2% prochloraz (Sportak), and placed in moist peat (60-80% w/w water content) in boxes. A polyethylene film (0.03-0.04 mm) with small holes was used to avoid drying of the content. The bulbs were acclimatized for 6 weeks at 2 □C and then placed at -2 □C (±0.1 □C).

Viability tests

In the autumn of 1993, the viability of bulbs stored for 2 to 4 years was tested and in the autumn of 1994 the viability of bulbs stored for 0 to 5 years was tested. Viability was determined by the proportion of bulbs with at least one regenerative scale (bulb regeneration), and the proportion of regenerative scales (scale regeneration). The sample sizes are shown in Table 4.1. The number of scales per bulb varied between 14 and 49. In the 1994 experiment, also ion leakage of scales was determined.

Table 4.1 The number of bulbs used to determine bulb and scale regeneration.

	Storage duration (years)					
Cultivar	**0**	**1**	**2**	**3**	**4**	**5**
Avignon	5	5	10	10	10	5
Connecticut King	5	5	5	5	5	5
Enchantment	5	5	10	10	10	5
Esther	5	5	10	10	10	5
Mont Blanc	5	5	10	5	5	5
Gelria	5	5	10	10	5	0
Snow Queen	5	5	10	10	10	5
Star Gazer	5	5	10	10	10	5

In regeneration tests, the scales were separated from the basal plate and dipped for 10 minutes in a mixture of 1% captan and 0.2% prochloraz (Sportak), to inhibit growth of micro organisms during propagation. Subsequently, scales were dried for 1 hour at room temperature and planted with the bottom down in moist peat in a greenhouse at 20 □C. Water was added regularly. Scale bulblet formation was recorded after eight weeks of incubation.

Ion leakage tests were carried out with one inner scale from each of 5 bulbs per storage duration. No ion leakage tests were carried out on visibly rotted scales. This excluded

all bulbs stored for 5 years and bulbs of some cultivars stored for 3 and 4 years (Table 4.2). Ion leakage test were carried out as previously described (Bonnier *et al.*, 1994). Scales were washed with tap water and stored overnight in a polyethylene bag at 18-20 □C to avoid leakage from fresh wounds. The following day each scale was initially washed in distilled water and placed in 100 ml distilled water for 1.5 h at 20 □C. Subsequently, the conductivity of the samples was measured using a digital conductivity meter (Philips PW9526 with electrode PW9514/60). The conductivity of each scale was corrected by subtracting the conductivity of the distilled water (1.85 $TS.cm^{-1}$) and divided by the fresh weight of the scale.

Statistical methods

The number of regenerating bulbs or scales in a sample is determined by the sample size (n) and the chance (*P*) for regeneration of each bulb (*P*b) or scale (*P*s) (binomial distribution). The chance of loosing a genotype with sample size n can be calculated by $(1-P)^n$. Regeneration was defined sufficient for propagation if the risk of losing 10 bulbs or 100 scales of a genotype was smaller than 0.01. For bulb regeneration $(1-Pb)^{10} < 0.01$ for $Pb > 0.37$ and for scale regeneration $(1-Ps)^{100} < 0.01$ for $Ps > 0.05$. Scale and bulb regeneration were regressed on storage duration using a generalized linear model for proportions, giving estimates and standard errors for bulb (*P*b) and scale regeneration (*P*s) per storage duration (Payne *et al.*, 1993). The maximum storage period was defined as the length of the storage period until Pb and Ps were estimated not significantly ($P < 0.05$) higher than 0.37 and 0.05 respectively.

Electrical conductivity values were log-transformed to obtain a normal distribution of standard errors. Significances between storage durations were calculated per cultivar for the log-transformed values using the protected LSD-test (Snedecor and Cochran, 1980). The maximum storage duration was defined as the length of the storage period until ion leakage of inner scales increased significantly.

Results and Discussion

Bulb regeneration

Bulb regeneration was 90 % for 'Gelria' and 100 % for all other cultivars stored for 2 years or less (Fig. 4.1A,B). With longer storage, bulb regeneration was lower for the *L.*

longiflorum cultivars and the Oriental hybrid 'Star Gazer' (Fig. 4.1B) than for the Asiatic hybrid cultivars (Fig. 4.1A). After 3 years, all *L. longiflorum* cultivars failed to regenerate and bulb regeneration of 'Star Gazer' was very poor. For the Asiatic hybrids 'Enchantment', 'Esther', and 'Mont Blanc', bulb regeneration remained 100 % for 3 years of storage. For 'Avignon' and 'Connecticut King' it was 25 % and 60 % respectively after 3 years of storage. After 4 years, bulb regeneration varied between 10 and 70 % for the Asiatic hybrid cultivars. No bulb regeneration was found after 5 years of storage.

Scale regeneration

Scale regeneration was approximately 100 % for nearly all tested non-stored bulbs (Fig. 4.2A,B). Loss of viability could be measured after 1 year. The earliest decline in viability was found for the *L. longiflorum* cultivars and for the Oriental hybrid 'Star Gazer' (Fig. 4.2B). After 3 years, only 15 and 3 % of the scales of the two regenerative 'Star Gazer' bulbs formed scale bulblets. For Asiatic hybrid cultivars (Fig. 4.2A), the variation in scale regeneration was high after 2 and 3 years of storage. Scale regeneration was low for most of the bulbs after 4 years, although a few bulbs of 'Enchantment' and 'Esther' had about 80 % regenerative scales (Fig. 4.2).

Bulb and scale regeneration are direct methods to measure viability of lily bulbs. Both should be measured to exclude incorrect conclusions based on a very high bulb regeneration with only a few scales per bulb forming scale bulblets, or on a relative high scale regeneration caused by only a few bulbs with high percentages of regenerating scales.

Figure 4.1 The relation between storage duration at -2 □C and the percentage of bulbs with at least one regenerative scale (bulb regeneration). Asiatic hybrids (**A**): 'Avignon' (—💻—), 'Connecticut King' (--❑--), 'Enchantment' (🗀 🗀 🗀 ✂ 🗀 🗀 🗀), 'Esther' (– – x – –), 'Mont Blanc' (– 🗀 🗀 🔭 – 🗀 🗀). Other genotypes (**B**): *L. longiflorum* 'Gelria' (—✂—), *L. longiflorum* 'Snow Queen' (--❶--), 'Star Gazer' (Oriental hybrid) (🗀 🗀 🗀

✎ 🖃 🖃 🖃). Bulb regeneration was regressed on storage duration using a generalized linear model for proportions. The number of bulbs used per storage duration and cultivar is presented in Table 4.1.

Figure 4.2 The relation between storage duration at -2 □C and the percentage of regenerative scales per bulb (scale regeneration). Asiatic hybrids (**A**): 'Avignon' (——), 'Connecticut King' (----), 'Enchantment' (□ □ □ □ □), 'Esther' (– – – –), 'Mont Blanc' (– □ □ – □ □). Other genotypes (**B**): *L. longiflorum* 'Gelria' (——), *L. longiflorum* 'Snow Queen' (----), 'Star Gazer' (Oriental hybrid) (□ □ □ □ □). Scale regeneration was regressed on storage duration using a generalized linear model for proportions. The vertical lines represent the differences

between the bulbs with maximum and minimum scale regeneration per observation for each genotype. The number of bulbs used per storage duration and cultivar is presented in Table 4.1.

Ion leakage

For all cultivars, electrical conductivity of external solution of inner scales did not increase until and including 2 years of storage (Table 4.2). However, after 3 years storage, all scales of the *L. longiflorum* cultivars had rotted, and electrical conductivity was increased for the other cultivars, except 'Enchantment' and 'Mont Blanc'. For 'Enchantment' and 'Mont Blanc', electrical conductivity was increased after 4 years storage. Inner scales lose viability later than other scales during storage of bulbs at -2 □C in moist peat (Bonnier *et al.*, 1994). Therefore, viability of inner scales can be used as a criterion to determine maximum storage duration of bulbs.

Table 4.2 Ion leakage, measured by electrical conductivity ($TS.cm^{-1}.g^{-1}$) of external solution of inner scales from eight lily cultivars stored 0 to 5 years at -2 □C. Each value represent the mean of five scales from 5 bulbs. Significances between storage durations were calculated with log-transformed values. The presented data are back-transformed means.

	Storage duration (years)								
Cultivar	**0**	**1**		**2**		**3**		**4**	
Avignon	0.41 a[1]	0.41	a	0.43	a	20.79	b	14.76	b
Connecticut King	0.65 a	0.23	a	*		3.90	b	**	
Enchantment	0.42 b	0.22	ab	0.12	a	0.60	b	3.45	c
Esther	0.27 a	0.25	a	0.27	a	1.05	b	3.56	c
Mont Blanc	0.38 a	0.33	a	0.28	a	0.65	a	**	
Gelria	0.16 a	0.21	a	0.28	a	**		**	
Snow Queen	0.31 a	0.25	a	0.24	a	**		**	
Star Gazer	0.55 a	0.39	a	0.38	a	2.08	b	**	

[1] the minor letters represent row significances ($P < 0.05$); * = not tested; ** = all scales had rotted.

Maximum storage duration

Based on bulb regeneration, the maximum storage duration was 2 years for 'Gelria' and slightly larger for 'Snow Queen' and 'Star Gazer' (Table 4.3). For Asiatic hybrids, it was between 2.9 e.g. 'Avignon' and 4 years e.g. 'Enchantment' and 'Esther'. For most cultivars, maximum storage duration based on scale regeneration was higher than maximum storage duration based on bulb regeneration. It was slightly lower for 'Connecticut King', and 'Star Gazer'. Maximum storage duration based on ion leakage was longer than 2 years for all cultivars and was shorter than 3 years except for 'Enchantment' and 'Mont Blanc' (Table 4.3). Unfortunately, bulbs of 'Connecticut King' after 2 years of storage were not available in the 1994 experiment. Therefore, ion leakage could not be tested for these bulbs.

Table 4.3 Maximum storage duration based on bulb regeneration (the chance of losing 10 bulbs was smaller than 0.01), scale regeneration (the chance of losing 100 scales was smaller than 0.01), and electrical conductivity (interval between the last year that the electrical conductivity was not increased and the first year that the electrical conductivity was increased significantly).

	Maximum storage duration (years) for		
Cultivar	**Bulb regeneration**	**Scale regeneration**	**Electrical conductivity**
Avignon	2.9	2.9	2-3
Connecticut King	3.4	3.3	1-3
Enchantment	4.0	5.0	3-4
Esther	4.0	4.5	2-3
Mont Blanc	3.4	3.9	3-*
Gelria	2.0	2.7	2-*
Snow Queen	2.2	3.0	2-*
Star Gazer	2.5	2.4	2-3

* = electrical conductivity was not determined, because all scales had rotted at longer storage durations than the years at left side of the interval.

A collection of lily genotypes contains a wide genetic variation. Therefore, maximum storage duration per group should be based on the genotypes that initially lose viability. The maximum storage duration for Asiatic hybrids was 2.9 years based on 'Avignon'. For the *L. longiflorum* cultivars and the Oriental hybrid, it was 2.0 years. Genetic variation was included in the experiment by using eight lily cultivars of diverse genetic backgrounds. Variation caused by different growing seasons was included by using bulbs grown in different seasons. Therefore, it appears likely, that also other lily genotypes of Asiatic hybrids, Oriental hybrids and *L. longiflorum* can be stored for 2 years at -2 □C in moist peat.

The maintenance of a lily collection can further be improved, if storage of bulbs in moist peat for 2 years is combined with storage of scale bulblets. Scale bulblets of lily can be stored for 2 years at -2 □C sealed air tight in low density polyethylene bags of 0.05 mm thickness (Bonnier *et al.*, 1996). Combining these storage methods would give the following maintenance scheme:

1) Storage of bulbs in moist peat for 2 years at -2 □C.
2) Regeneration of scale bulblets.
3) Storage of scale bulblets sealed in polyethylene bags for 2 years at -2 □C.
4) Production of larger bulbs in the field from the scale bulblets for 2 years.

Using this maintenance scheme, bulbs only need to be planted in the field twice in 6 years.

Chapter 5

Long term lily scale bulblet storage: effects of temperature and storage in polyethylene bags

Bonnier, F.J.M, R.C. Jansen, and J.M. van Tuyl, 1996. Annals of Applied Biology 129: 161-169.

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Abstract

Collections of lily genotypes are usually maintained by yearly planting, harvesting and storage of the bulbs. To facilitate this maintenance, a storage method has been developed for a collection of lily genotypes, including Asiatic hybrids, Oriental hybrids, *Lilium longiflorum*, and *L. henryi*. Scale bulblets were stored either dry, sealed air-tight in polyethylene bags, or in moist vermiculite in open polyethylene bags for a period of 2 years. The decrease in mass, sprouting proportion and ion leakage or sprouting proportion alone were determined for treatments carried out at -2 □C, 0 □C and 17 □C. Sealing scale bulblets in polyethylene bags at -2 □C resulted in the smallest decrease in mass, the least ion leakage and the highest sprouting proportion after 2 years of storage.

Introduction

To support breeding and research, the Centre for Plant Breeding and Reproduction Research (CPRO-DLO) maintains a lily collection of more than 1000 genotypes. Collections of clonal material are usually propagated by yearly planting, harvesting and storage, which involves a considerable investment in labour and space and also introduces the risk of losses through disease (Withers, 1991). Methods for long term storage of vegetative material would make maintenance more efficient. Techniques for long-term storage of clonal material should create conditions for slow growth or use cryopreservation (Grout, 1991; Towill, 1988; Withers, 1991). The most easy way to minimize growth is storage at low temperature. However, this is limited by the sensitivity to frost damage of lily bulbs. Lily bulbs can be stored at -2 □C to obtain flowering out of season (Boontjes, 1983; Beattie and White, 1993; Bonnier *et al.*, 1994).

As well as low temperatures, the rate of metabolism of the material can also be decreased by enriching the atmosphere with CO_2 and decreasing the partial pressure of O_2 under controlled conditions, called controlled atmosphere storage (CAS). Storage under low partial pressure of oxygen may also increase frost tolerance, since an inverse relationship was found between frost survival and atmospheric pressure for five plant and two vertebrate species (Halloy and González, 1993). Modified atmosphere (MA) packaging in polyethylene film bags has been used to extend the storage life of many crops including pre-cooled tulip bulbs (Prince *et al.*, 1981; Prince *et al.*, 1986). An atmospheric equilibrium develops in the bags, which is enriched in CO_2 and diminished in O_2. The equilibrium is dependent on the respiratory rate of the material and the gas-permeability of the bags (Prince *et a*l., 1986). If the temperature decreases, both the respiration of the tissue and the gas-permeability of the bags decrease (Prince *et al.*, 1986). Sealing in gas-permeable bags has several advantages over storage in rooms with a controlled gas composition. Firstly, material is divided into small batches, reducing the risk of quickly spreading diseases. Secondly, gas-permeable bags are much cheaper than CAS. However, one disadvantage is that the atmosphere is less accurately controlled. MA packages have also been shown to create a suitable environment for infection by fungi and bacteria (Prince *et al.*, 1987). Therefore, material needs to be disinfected before sealing.

Lily bulbs can be propagated by regeneration of scale bulblets on detached lily scales (Van Tuyl, 1983; Matsuo and Van Tuyl, 1984). Regeneration of lily scale bulblets on scales appeared to be possible after 2 to 3 years storage of bulbs at -2 □C in moist peat (Bonnier *et al.*, 1994). Combining storage of bulbs and storage of scale bulblets would further reduce the frequency bulbs need to be planted in the field. Ion leakage has been shown to be a useful criterion to measure viability of lily scales (Bonnier *et al.*, 1994). In this study, storage of lily scale bulblets in open and closed polyethylene bags was investigated. Viability was measured by the sprouting proportion and by ion leakage. Ion leakage was tested as a criterion to measure viability of scale bulblets.

Material and methods

Plant material

Lily genotypes representing Asiatic hybrids ('Avignon', 'Connecticut King', 'Enchantment', 'Esther', and 'Mont Blanc'), Oriental hybrids ('Casa Blanca', and 'Star Gazer'), *L. longiflorum* ('Gelria', and 'Snow Queen'), and *L. henryi* (accession no. 72122) were used. For each genotype, scales from 10 bulbs (circumference 12-16 cm) were detached for propagation. Scales were dipped in fungicide for 10 minutes in 1 $g.l^{-1}$ captan and 0.2 $g.l^{-1}$ prochloraz (Sportak), surface-dried for 1 h, planted in moist vermiculite and placed for 8 weeks at 25 □C and 4 weeks at 17 □C, to regenerate scale bulblets (Van Tuyl, 1983). Scale bulblets were subsequently removed from the scales and disinfected again.

Scale bulblets were randomized for each genotype and put into groups of five. For each group the fresh weight was measured and the scale bulblets were then either sealed dry in low-density polyethylene bags, or placed in moist vermiculite in open polyethylene bags, and stored at -2 □C, 0 □C and 17 □C (±0.1 □C). The low-density polyethylene bags (Stibbe bv, Zwolle, The Netherlands) were 11 cm x 12 cm, and had a thickness of 0.05 mm. During storage, the total air volume in the sealed bags decreased until a vacuum was established within 4 months to 1 year, dependent on the size of the bulblets and the temperature.

For scale bulblets sealed in polyethylene bags, decrease in mass, sprouting proportion, and ion leakage were recorded every 4 months, during the 2 years storage. After storage for 1 year, it was noticed that the scale bulblets were drying. The sealed bags were

then placed under conditions of 100 % relative humidity in a box with moist vermiculite, to prevent further drying out. For scale bulblets stored in open bags in moist vermiculite, only sprouting proportion was observed. Ion leakage and decrease in mass could not be determined, because of the occurrence of rotten scale bulblets in the vermiculite. Scale bulblets of Oriental hybrids, *L. longiflorum* and *L. henryi* were scored only 6 ('Gelria'), 5 ('Casa Blanca), 5 ('Snow Queen), 4 ('Star Gazer'), and 3 (*L. henryi*) times, because of a limited availability of scale bulblets of these genotypes.

Decrease in mass

The sealed bags containing the scale bulblets were weighed before and after storage. The decrease in mass was calculated with respect to the initial fresh mass of the scale bulblets.

Ion leakage

The method of measuring ion leakage of lily scale bulblets by conductivity was modified from that used to measure ion leakage of lily scales (Bonnier *et al.*, 1992; Bonnier *et al.*, 1994). The roots of the scale bulblets were removed, and after one night at 20 □C, groups of five scale bulblets were placed for 1.5 h in 100 ml of distilled water at 20 □C. Subsequently, the electrical conductivity of the samples was measured with a digital conductivity meter (Philips PW9526 with electrode PW9514/60) and each sample corrected by subtracting the conductivity of a distilled water control and dividing by the original fresh weight of the scale bulblets.

Sprouting proportion

After measuring conductivity, the same scale bulblets were planted in soil. Those stored at -2 □C and 0 □C were placed directly in a greenhouse. The scale bulblets stored at 17 □C were first placed at 5 □C for 12 weeks (to break dormancy) and then cultured in the greenhouse. After 6 weeks, the sprouting proportion of the scale bulblets was recorded. After a storage duration of 2 years, the sprouting scale bulblets were kept in the greenhouse for 0.25 years to check their ability to form new bulblets. All sprouting scale bulblets formed new bulblets.

Table 5.1 Means of initial mass, decrease in mass (percentage of initial mass and angular transformed values), conductivity (original values and log-transformed values), and sprouting proportions (predictions from generalized linear model for proportions + standard errors) of two groups of five lily scale bulblets sealed in polyethylene bags, after storage at -2 □C for 2 years.

Genotype	Mean initial mass (g)	Proportion of initial mass (%)	(angle)	Conductivity ($TS.cm^{-1}.g^{-1}$) (mean)	(ln)	Sprouting proportion (predicted) (%)	(se)
Avignon	0.80	92	73.7	0.83	-0.19	94.9	3.6
Connecticut King	0.79	89	70.3	1.19	0.17	100.0	0.0
Enchantment	0.46	84	66.7	0.76	-0.28	97.0	2.2
Esther	1.28	96	78.4	0.51	-0.67	100.0	0.0
Mont Blanc	0.60	90	71.4	1.19	0.17	100.0	0.0
Casa Blanca	1.25	94	76.3	0.86	-0.15	62.1	8.2
Star Gazer	2.20	97	79.6	0.52	-0.66	92.9	4.2
Gelria	0.80	90	72.0	1.27	0.24	93.5	3.7
Snow Queen	0.74	92	73.5	0.28	-1.28	100.0	0.0
L. henryi	1.22	92	73.4	0.71	-0.34	74.6	7.5
LSD ($P \leq 0.05$)			5.4		N.S.[z]		

[z] N.S. = no significances

Statistical methods

Decrease in fresh mass was fitted exponentially on storage duration ($y = A + B*R^X$) per temperature and then expressed on initial mass base (Fig. 5.1). Significance levels between cultivars after 2 years storage at -2 □C (Table 5.1) and between storage durations per temperature (results in text) were calculated using the protected LSD-test after angular transformation of proportions (Snedecor and Cochran, 1980).

Conductivity values were log-transformed to obtain a normal distribution of standard errors. Natural logarithms of conductivity values were fitted linearly on storage duration for the temperatures -2 □C and 0 □C and by a gompertz curve (y = A + C*EXP(EXP(-B*(X-M)))) (Payne *et al.*, 1993) for the temperature 17 □C (Fig. 5.2). Significances between cultivars after 2 years of sealed storage at -2 □C (Table 5.1) were calculated for the log-transformed values using the protected LSD-test (Snedecor and Cochran, 1980).

The sprouting proportions of the scale bulblets (Fig. 5.3A,B) were regressed per treatment on storage duration using a generalized linear model for proportions (Payne *et al.*, 1993). For the treatment with the highest sprouting proportion after 2 years: decrease in mass, conductivity values and predicted sprouting proportions were summarized per genotype (Table 5.1).

Results

Decrease in mass

During the first year of dry storage, the sealed scale bulblets lost mass. The decrease was relatively small at -2 □C, but at 17 □C, the decrease in mass was about 70%. After transfer of the sealed bags to conditions of 100 % relative humidity after 1 year storage, no further significant decrease in mass was found (Fig. 5.1). Differences between genotypes were mainly caused by differences in the original mass of the scale bulblets. Genotypes with light scale bulblets lost mass quicker than genotypes with heavier scale bulblets. The correlation coefficient between initial mass and mean loss of mass was 0.93 for scale bulblets stored for 2 years at 0 □C.

Ion leakage

Electrical conductivity values of sealed scale bulblets stored at -2 □C were low and no increase in ion leakage was observed during storage (Fig. 5.2). At the end of the storage period electrical conductivity was still below 1 $TS.cm^{-1}.g^{-1}$ for all genotypes (Table 5.1). The mean electrical conductivity of sealed scale bulblets stored at 0 □C was higher than at -2 □C after 2 years of storage, but the increase in conductivity at 0 □C was small and not significant (Fig. 5.2). During storage at 17 □C, electrical conductivity increased relatively quickly. The increase was significant after storage for 0.67 years and longer.

Figure 5.1 Mean and fitted decrease in fresh mass for scale bulblets of 10 genotypes (2 x 5 scale bulblets of each) during storage in sealed polyethylene bags at -2 □C (), 0 □C (❑) and 17 □C (). Decrease in fresh mass was fitted exponentially per temperature ($y = A + B * R^X$) and then expressed on initial mass base. The vertical lines represent standard errors per observation.

Figure 5.2 Mean and fitted electrical conductivity values for scale bulblets of 10 genotypes (2 x 5 scale bulblets of each) during storage in sealed polyethylene bags at -2 □C (), 0 □C (❑) and 17 □C (). Natural logarithms of conductivity values were fitted linearly on storage duration for the temperatures -2 □C and 0 □C and by a gompertz curve ($y = A + C * EXP(EXP(-B * (X-M)))$) for the temperature 17 □C. The vertical lines represent standard errors per observation at -2 □C, 0 □C and 17 □C respectively.

Figure 5.3 Mean and fitted sprouting proportions for scale bulblets of 10 genotypes (2 x 5 scale bulblets of each) during dry storage in sealed polyethylene bags (A) and storage in moist vermiculite in open polyethylene bags (B) at

-2 □C (🖎), 0 □C (❑) and 17 □C (💻). Sprouting proportions were fitted on storage duration using a generalized linear model for proportions. The vertical lines represent the differences between the genotypes with maximum and minimum sprouting proportion per observation at -2 □C, 0 □C and 17 □C respectively.

Sprouting proportion

After storage for 2 years, the highest sprouting proportion was found for sealed scale bulblets stored at -2 □C (Fig. 5.3A). The two genotypes with the lowest predicted sprouting proportion were *L. henryi* and 'Casa Blanca' (Table 5.1). Predictions for their sprouting proportion and standard error indicate that storage is possible for longer than 2 years. Sealed storage at 0 □C was less effective than that at -2 □C (Fig. 5.3A). The predicted sprouting proportion of *L. henryi* was not significantly different from zero after 2 years storage at 0 □C (results not shown). A relatively quick decrease in sprouting proportion was observed for storage in sealed polyethylene bags at 17 □C (Fig. 5.3A). This was probably caused by drying out of the scale bulblets (Fig. 5.1).

A quicker decline in the sprouting proportion was found for unsealed rather than sealed storage at -2 □C (Fig. 5.3A,B). During unsealed storage at -2 □C, soft brown scale bulblets were often observed for genotypes of Oriental hybrids, *L. longiflorum* and *L. henryi*. Scale bulblets stored unsealed at 0 □C and 17 □C often started sprouting during storage. The sprouting proportion declined in an almost equal rate during storage for both treatments. Unsealed storage at 0 □C and 17 □C was less effective than that at -2 □C (Fig. 5.3B).

Discussion

Sealing scale bulblets in polyethylene bags at -2 □C resulted in the least decrease in mass, the highest sprouting proportion and the least ion leakage after 2 years of storage. Storage for longer than 2 years appears possible. Storage of scale bulblets sealed in polyethylene bags at 17 □C resulted in relatively quick drying out of the scale bulblets, leading to a quick rise in conductivity values and a fast decline in sprouting proportion. Sealed storage at 17 □C would probably have scored better, if the scale bulblets had been protected against drying out from the beginning by placing the polyethylene bags under conditions of 100 % relative humidity.

Prince *et al.* (1986) found gaseous equilibrium levels of both O_2 and CO_2 between 3 and 5 % for tulip bulbs stored in a low-density polyethylene package. The equilibrium levels were hardly affected by temperature. The level for N_2 was not stated, but it must have been between 90 and 94 %. Under such circumstances, N_2 would diffuse out of the package. We

did not measure gaseous levels in the packages, but we used a similar low-density polyethylene film for lily scale bulblets at -2, 0, and 17 □C. Therefore, the establishment of a vacuum in the sealed polyethylene bags was probably caused by diffusion of N_2 from the package during storage.

During unsealed storage at -2 □C, decrease in sprouting proportion of genotypes of the Oriental hybrids, *L. longiflorum* and for *L. henryi* was probably due to injury caused by freezing, since soft brown scale bulblets were observed and genotypes of Oriental hybrids and *L. longiflorum* are known to be less freeze tolerant than Asiatic hybrids (Boontjes, 1983). The scale bulblets sealed in polyethylene bags were more tolerant to freezing, which can be explained in two ways. Firstly, a reduced oxygen pressure in the bags might have increased freezing tolerance, supporting the results of Halloy and González (1993). Secondly, the scale bulblets sealed in polyethylene bags were partly dried out. It is known that a lower water content increases freezing tolerance (Lång *et al.*, 1994; Maier *et al.*, 1994; Pearson and Davison, 1994). The ability to store partially dried material is therefore an advantage of storing scale bulblets in sealed polyethylene bags.

Scale bulblets stored unsealed at 0 □C and 17 □C, tended to sprout during storage, which probably exhausted the carbohydrate resources and reduced their viability. Scale bulblets stored dry, and sealed in polyethylene bags, did not sprout during storage at 0 □C and 17 □C, probably because of lack of water. The prevention of sprouting in the package is another advantage of storing in this way.

When tissue dies, cell membranes detoriate and the tissue starts to leak nutrients. Therefore, a negative correlation was expected between electrical conductivity values and sprouting proportions. During sealed storage at 17 □C, an increase in electrical conductivity values was accompanied by a decrease in sprouting. However, during sealed storage at -2 □C and 0 □C a decline of the sprouting proportion was often recorded without an increase in electrical conductivity. This might be explained by a different viability of the sprouts and the scales of the scale bulblets. The sprouting proportion is determined by the health status of the meristems, whereas ion leakage is determined by the health status of the scales. If the meristem in a scale bulblet is damaged and the surrounding scales of the scale bulblet are still healthy, the scale bulblet will not sprout, but will leak few ions. Ion leakage was therefore concluded to be a less useful criterion to measure the viability of lily scale bulblets than that of lily scales (Bonnier *et al.*, 1994).

The maintenance of a lily collection can further be improved, if storage of scale bulblets for 2 years is combined with storage of larger (circumference between 12 and 16 cm) bulbs in moist peat. Bulbs of Asiatic hybrids can be stored for 3 years and bulbs of Oriental hybrids and genotypes of *L. longiflorum* for 2 years in moist peat (Bonnier and Van Tuyl, 1996). Combining these storage methods gives the maintenance scheme presented on page 34. Bulbs will only need to be planted in the field twice in 6 years, using this maintenance scheme.

The storage methods used in the present study were tested using 10 lily genotypes. Since the genetic backgrounds of the genotypes included a wide genetic variation, it is likely, that storage at -2 □C in polyethylene bags is also suitable for other genotypes of lily.

Chapter 6

Long term *in vitro* storage of lily: effects of temperature and concentration of nutrients and sucrose

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Abstract

Methods for long term preservation of lily germplasm were examined. *In vitro* regenerated bulblets of 10 lily (*Lilium* L.) genotypes (Asiatic hybrids, Oriental hybrids, *L. longiflorum* and *L. henryi*) were stored for 28 months at -2 □C and 25 □C on four different media: 1/4 or full strength Murashige and Skoog nutrients with 9 % or 6 % (w/v) sucrose. Sprout growth, bulb growth, and viability were determined. The combination of 1/4 strength MS nutrients and 9 % sucrose gave the highest reduction in sprout and bulb growth, the highest viability and the highest percentage of regrowth after 28 months of storage. At 25 □C, all lily genotypes survived 28 months of storage under these conditions. At -2 □C, Asiatic and Oriental hybrids survived 28 months of storage, whereas genotypes of *L. longiflorum* and *L. henryi* survived 6 months of storage, but died during prolonged storage.

Introduction

Genetic improvement of crops depends on available genetic diversity. Therefore, lily breeding companies and research institutes preserve collections of lily genotypes. To make this preservation more efficient, techniques are being developed for long term storage of vegetative material of lily (Bonnier *et al.*, 1992; Bonnier *et al.*, 1994; Bonnier and Van Tuyl, 1996; Bonnier *et al.*, 1996). The maintenance of a germplasm collection in vitro has several advantages over storage of bulbs, as it requires small amounts of space and it reduces the risk of losses caused by diseases (Towill, 1988).

For long-term *in vitro* storage, clonal material can be kept under conditions for slow growth or it can be cryopreserved (Grout, 1991; Towill, 1988; Withers, 1991). Lily meristems have been successfully cryopreserved, by slow freezing (Bouman and De Klerk, 1990) and vitrification (Matsumoto *et al.*, 1995). Yet, cryopreservation requires methods to enhance dehydration or freezing tolerance, that appeared to be genotype-specific (Matsumoto *et al.*, 1995).

Slow growth *in vitro* may be obtained by low temperature, osmotic stress (Grout, 1991; Withers, 1991), or a low concentration of nutrients (Engelmann, 1991). Low temperature though is not directly applicable to lily because, at low temperature (0-10 □C), dormancy of the bulblets is broken and the bulblets sprout during prolonged storage (De Klerk and Paffen, 1995; Higgins and Stimart, 1990; Kawarabayashi, 1995). A temperature of -2 □C is commonly used to store lily bulbs (Beattie and White, 1993; Bonnier and Van Tuyl, 1996).

Because dormant bulblets have an increased resistance to environmental stress (De Klerk and Gerrits, 1996), they may be stored for a prolonged period. A temperature of 25 □C induces dormancy in different lily species and hybrid groups (Aguettaz *et al.*, 1990; De Klerk and Paffen, 1995; Delvallée *et al.*, 1990; Gerrits and De Klerk, 1992; Gerrits *et al.*, 1995; Kawarabayashi, 1995).

We examined the effect of tissue culture conditions on bulb growth, sprout growth and viability of lily *in vitro* bulblets during prolonged storage. We used low temperature (-2 □C) or a dormancy inducing temperature (25 □C), in combination with a low nutrient and a high sucrose concentration in the medium.

Material and methods

Plant material

The cultivars used for the experiment were: 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Asiatic hybrids), 'Casa Blanca', 'Star Gazer' (Oriental hybrids), 'Gelria', 'Snow Queen' (*L. longiflorum*) and *L. henryi* (CPRO-number 72122). The genotypes were established into *in vitro* conditions by PermX Multiplant, a former commercial tissue culture company in Wageningen. Scales from bulbs with a circumference between 12 and 16 cm were rinsed with tap water and surface-sterilized for 30 minutes in 1 $g.l^{-1}$ NaOCl. Scales were then washed in sterile deionised water and scale segments of about 7 x 7 mm were placed with the abaxial side on MS medium (Murashige and Skoog, 1962) with 10 $g.l^{-1}$ purified agar (Sigma) and 6 % (w/v) sucrose in glass tubes (2.2 cm diameter). The scale segments were cultured at 25 □C with 16-hour day light at an intensity of 10-20 $TE.m^{-2}.s^{-1}$ (Philips TLD 36W/84). After two months, scale bulblets were cut from the scale segments given the various treatments in glass tubes (2.2 cm diameter) containing 20 ml medium.

Treatments

Four different media with 10 $g.l^{-1}$ purified agar (Sigma) were used: a decreased (1/4 strength) or standard concentration MS nutrients and an increased 9 % (w/v) or standard 6 % (w/v) concentration of sucrose. The glass tubes were closed by a glass cover. A polyethylene film was wrapped around the glass cover to protect the media and bulblets from drying. The glass tubes were placed at 25 □C with 16-hour day light at an intensity of 10-20 $TE.m^{-2}.s^{-1}$ (Philips TLD 36W/84). The time at which the scale bulblets were placed on the different media was regarded as the start of storage. After 4 months of storage, we received the scale bulblets from PermX Multiplant. Sprout length, bulblet thickness and viability was determined, and afterwards one-half of the tubes were placed at +2 □C for 2 weeks for acclimatization, and then transferred to -2 □C in the dark. The other one-half of the tubes were placed at 25 □C again under conditions of low light (16-hour day, 0.01-0.1 $TE.m^{-2}.s^{-1}$). Per treatment, 13-20 scale bulblets were used, with exception of 'Esther' (3-7), 'Snow Queen' (3-14), and *L. henryi* (3-9).

Sprout length

The length between the bottom of the bulblets and the top of the sprouts was measured after 4 months storage, before transferring one-half of the tubes to +2 □C. Sprout length was not measured at longer storage durations, because sprouts in some tubes had reached the top of the tube at the first measurement. Therefore, sprout growth at -2 □C was not observed. Differences between treatments were analyzed in couples using the rank sum Mann-Whitney test for two independent samples (Snedecor and Cochran, 1980).

Bulblet growth

After 4 and 10 months storage, the largest diameter of each scale bulblet was measured. Diameter at 10 months divided by diameter at 4 months was used as a measure for bulblet growth. The data for bulblet growth were transformed by natural logarithm to obtain a normal distribution. Differences between treatments were analyzed by F-test followed by LSD.

Visual viability assessment

Viability was assessed when the bulblets were received after 4 months of storage and, thereafter, every 6 months by visually estimating the proportion of healthy-looking tissue per tube. Viability was regressed per treatment on storage duration using a generalized linear model for proportions (Payne *et al.*, 1993). The effect of the interaction between medium and genotype appeared to be small (deviance ratio = 5,59) compared to the effects of storage time (deviance ratio = 4447), genotype (deviance ratio = 294.0) and medium (deviance ratio = 74.02). Therefore, viability of bulblets was regressed per medium for all genotypes, and per group of genotypes for all media on storage duration for both treatments at -2 □C and 25 □C.

Growth after storage

After 28 months storage, the bulblets that were stored at -2 □C were acclimatized at 5 □C for one week. Then, bulblets from all treatments were taken from the tubes and planted

in moist peat. The bulblets from cold storage treatment were directly placed in a greenhouse at 20 □C. The bulblets that were stored at 25 □C were first placed for 8 weeks (Asiatic hybrids, *L. longiflorum*, and *L. henryi*) or 12 weeks (Oriental hybrids) at 5 □C, to break dormancy, and then placed in the greenhouse at 20 □C. The percentage of bulblets forming new bulblets after sprouting was calculated for all treatments. Differences between media and storage temperatures were analyzed using a generalized linear model for proportions (Payne *et al.*, 1993).

Correlations between assessments

Spearman's rank correlation coefficients (r_s) (Snedecor and Cochran, 1980) were calculated from ranks per treatment to determine the relation of sprout and bulblet growth with viability and regrowth, and to determine the relation between visual viability assessment and regrowth.

Results

Table 6.1 Length of *in vitro* lily sprouts (cm) after 4 months growth at 25 □C on four different growth media. Significant differences between rows are represented by small letters ($P < 0.05$) and capitals ($P < 0.01$).

Genotype	**MS Medium**			
	1/1 nutrients 6% sucrose	**1/1 nutrients 9% sucrose**	**1/4 nutrients 6% sucrose**	**1/4 nutrients 9% sucrose**
Asiatic hybrids	8.6 a	5.9 b	2.8 c	2.4 c
Oriental hybrids	1.4 ab	1.8 a	1.1 c	1.2 bc
L. longiflorum	9.4 a	4.5 b	2.8 c	1.7 c
L. henryi	6.8 a	3.3 b	1.4 c	1.4 c
Means	7.0 A	4.5 B	2.3 C	1.9 C

Sprout length

Sprout growth at 25 □C was significantly reduced by using 1/4 strength MS medium (Table 6.1). Also, an increase of the sucrose concentration from 6 to 9 % reduced sprout growth, but the effect was smaller and not significant for the low nutrient medium. The effects were generally similar for all genotypes, except for the Oriental hybrids. Both the Oriental hybrids 'Casa Blanca' and 'Star Gazer' hardly formed sprouts on any of the four growth media.

Bulblet growth

Bulblet growth at 25 □C was significantly reduced by using 1/4 strength MS medium (Table 6.2). For all groups, the effect of the sucrose concentration was not significant. The interaction between effects of growth medium and genotype was significant but ten times smaller than main effects of growth medium and genotype, indicating a nearly similar behaviour of genotypes. At -2 □C, bulblet growth was very low and no significant differences were found between treatments (results not shown).

Table 6.2 Bulblet growth of *in vitro* lily plants on four different growth media: largest diameter after 10 months growth at 25 □C divided by the largest diameter after 4 months growth at 25 □C. Significant differences between rows are represented by small letters ($P < 0.05$) and capitals ($P < 0.01$).

Genotype	**MS Medium**			
	1/1 nutrients 6% sucrose	**1/1 nutrients 9% sucrose**	**1/4 nutrients 6% sucrose**	**1/4 nutrients 9% sucrose**
Asiatic hybrids	2.12 a	2.08 a	1.63 b	1.60 b
Oriental hybrids	1.78 a	1.91 a	1.46 b	1.49 b
L. longiflorum	1.20 ab	1.33 a	1.11 b	1.20 ab
L. henryi	1.77 a	1.87 a	1.21 b	1.42 b
Means	1.80 A	1.87 A	1.48 B	1.48 B

Visual viability assessment

Both at -2 □C (Fig. 6.1A) and 25 □C (Fig. 6.1B), the highest viability was observed on the growth medium with 1/4 strength nutrients and 9 % sucrose; the lowest viability was found on the growth medium with standard nutrients and 6 % sucrose. Although the effect of growth medium was similar at -2 □C and 25 □C, the type of damage was quite different at both temperatures. At -2 □C, the growth medium in several tubes froze, leading to death of plants. Also, plants died of frost damage without freezing of the media, while other plants looked completely healthy. This frost damage was only observed at a storage duration of 16 months or longer and was absent at a storage duration of 10 months, when tubes were stored for one-half a year at -2 □C. Genotypes of *L. longiflorum* and *L. henryi* were relatively sensitive to frost damage (Fig. 6.2A). At 25 □C, browning of outer scales was observed, while inner scales remained viable. At -2 □C and at 25 □C, viability as measured by visual inspection was in the following descending order: Oriental hybrids, Asiatic hybrids, *L. henryi*, *L. longiflorum* (Fig. 6.2B).

Growth after storage

At -2 □C and 25 □C, the highest percentage of regrowth after 28 months of storage was observed on the growth medium with 1/4 strength of the standard nutrients plus 9 % sucrose and the lowest regrowth percentage on the growth medium with standard nutrients plus 6 % sucrose (Table 6.3). The interaction between effects of growth medium and temperature was not significant. Regrowth after storage at 25 □C was significantly ($P < 0.01$) higher than regrowth after storage at -2 □C. None of the genotypes of *L. longiflorum* and *L. henryi* survived 28 months of storage at -2 □C. Also regrowth of the Asiatic hybrid 'Mont Blanc' was beneath 10 % after storage at -2 □C on the 1/4 strength nutrient medium (result not shown). The mean regrowth percentage after 28 months storage at 25 □C on 1/4 strength MS medium plus 9 % sucrose was 92 % (Table 6.3) with a minimum of 61 % for *L. longiflorum* 'Gelria' (data not presented).

Correlations between assessments

Sprout length was significantly ($P < 0.05$) negatively correlated with visually assessed viability ($r_s = -0.45$) and regrowth percentage ($r_s = -0.32$).

Correlations between bulb growth and viability and regrowth were not significant. Visually assessed viability was significantly ($P < 0.01$) and positively correlated to regrowth ($r_s = 0.53$).

Figure 6.1 Mean (points) and regressed (lines) visually assessed viability on a scale from 1 (no visual damage) to 0 (dead) of 10 lily genotypes stored on four different growth media at -2 □C (**A**) and 25 □C (**B**): 1) MS medium plus 6 % sucrose (1/1 + 6); 2) MS medium plus 9 % sucrose (1/1 + 9); 3) 1/4 strength MS medium plus 6 % sucrose (1/4 +

6); 4) 1/4 strength MS medium plus 9 % sucrose (1/4 + 9). The LSD represent significant differences between media at a storage duration of 28 months ($P < 0.01$).

Figure 6.2 Mean (points) and regressed (lines) visually assessed viability on a scale from 1 (no visual damage) to 0 (dead) of 4 groups of lily genotypes stored on four different growth media at -2 □C (**A**) and 25 □C (**B**). Asiatic hybrids: 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc'; Oriental hybrids: 'Casa Blanca', 'Star

Gazer'; *L. longiflorum*: 'Gelria', 'Snow Queen'; *L. henryi*: clone 72122. The vertical lines represent a 99 % confidence interval for each genotype group at a storage duration of 28 months.

Table 6.3 Percentage of *in vitro* lily plants forming sprouts and new bulblets after transfer from a glass tube to a greenhouse. The plants were grown for 28 months on four different growth media at two storage temperatures. The small letters represent significant differences between rows ($P < 0.05$).

Storage temp.	Genotype	MS Medium			
		1/1 nutrients 6% sucrose	**1/1 nutrients 9% sucrose**	**1/4 nutrients 6% sucrose**	**1/4 nutrients 9% sucrose**
-2 □C	Asiatic hybrids	32	54	61	73
	Oriental hybrids	23	53	59	90
	L. longiflorum	0	0	0	0
	L. henryi	0	0	0	0
	Means	21 a	38 ab	42 ab	55 b
25 □C	Asiatic hybrids	84	87	98	97
	Oriental hybrids	64	78	84	97
	L. longiflorum	44	72	57	71
	L. henryi	0	33	40	100
	Means	63 a	77 ab	81 ab	92 b

Discussion

Storage at 25 □C on 1/4 strength MS medium plus 9 % sucrose resulted in the highest visually assessed viability and the highest regrowth percentage. All lily genotypes survived 28 months of storage under these conditions. The research was ended after 28

months of storage, but the results indicate that longer storage than 28 months was possible. Because the genotypes were from different hybrid groups and species, it seems likely, that other lily genotypes of Asiatic hybrids, Oriental hybrids and *L. longiflorum* can be stored *in vitro* for at least 28 months.

Reducing the concentration of MS nutrients in the medium to 1/4 strength led to a significant reduction in lily sprout and bulblet growth at 25 □C. Plants hardly sprouted in the 1/4 strength nutrient media, suggesting induction of dormancy. However, Aguettaz *et al.* (1990) found no effect of the concentration of MS nutrients on dormancy of *in vitro* plants of *L. speciosum*, while there was also no effect on the dry matter partitioning between leafs and bulblets of *in vitro* plants of *L. speciosum* (Gerrits and De Klerk, 1992). On full strength MS medium, 9 % sucrose instead of 6 % sucrose reduced sprout growth, but did not affect bulb growth at 25 □C. On 1/4 strength MS medium, the growth reducing effect of 9 % sucrose was not observed, probably due to the large growth reducing effect of the low nutrient concentration. A high concentration of sucrose can reduce growth by osmotic stress (Grout, 1991; Withers, 1991). It also promotes bulb formation in *L. auratum* (Takayama and Misawa, 1980), *L. speciosum* (Gerrits and De Klerk, 1992), and *Allium* species (Keller, 1991).

Slow growth increases the maximum storage duration (Grout, 1991; Withers, 1991). Therefore, sprout and bulblet growth were expected to be negatively correlated with visually assessed viability and regrowth. These negative correlations were found for sprout growth, but not for bulblet growth. This difference might be explained by the fact that during bulblet growth energy is placed into a storage organ, where it remains available for regrowth. During sprout growth, however, energy is removed from the medium and the bulblet.

A temperature of 25 □C induces dormancy in different lily species and groups (Aguettaz *et al.*, 1990; De Klerk and Paffen, 1995; Delvallée *et al.*, 1990; Gerrits and De Klerk, 1992; Gerrits *et al.*, 1995; Kawarabayashi, 1995). Yet, sprouting was observed at this temperature on MS medium plus 6 % sucrose for all genotypes, except the Oriental hybrids. Maybe light induced sprouting, as was found for *in vitro* plants of *L. speciosum* (De Klerk and Paffen, 1995). However the light intensity was very low (16-hours.day^{-1}, 0.01-0.1 TE.m^{-2}.s^{-1}).

At -2 □C, bulblets of *L. longiflorum* and *L. henryi* died during storage. A temperature of -2 □C is commonly used to store lily bulbs (Beattie and White, 1993, Bonnier and Van Tuyl, 1996) and for *in vitro* shoots and bulblets of *Allium* species -2 □C gave identical

results as +2 □C (Keller, 1992). Therefore -2 □C was expected to be a safe temperature to store *in vitro* bulblets of lily. The acclimatization period of two weeks was shorter than the period that is generally used to acclimatize bulbs (Beattie and White, 1993) to avoid further sprout growth of the bulblets. Insufficient acclimatization would immediately have resulted in damage. However, damage became only visible at storage durations of more than 6 months at -2 □C, indicating that freezing tolerance of *in vitro* plants was reduced during storage. Ice crystallization of the media in some glass tubes was another feature that was observed during storage at -2 □C. The crystallization seemed to be randomly divided over the different media and genotypes, leading to death of plants. These observations suggest that the media in the tubes were supercooled. Therefore, we conclude that -2 □C was too low for long term storage of *in vitro* lily bulblets. A slightly higher temperature than -2 □C might be a favourable storage temperature for lily *in vitro* germplasm, but might also induce leaf formation by breaking dormancy (De Klerk and Paffen, 1995: Higgins and Stimart, 1990; Kawarabayashi, 1995).

Visually assessed viability allowed to see the effect of the various conditions in time. It was positively correlated with regrowth percentage. This correlation was not expected to be high, because regrowth might still be 100 % when only a small proportion of tissue per tube looks healthy.

We conclude that storage of lily bulblets at 25 □C on 1/4 strength MS medium plus 9 % sucrose is a promising method to preserve a lily germplasm collection.

Chapter 7

Freezing tolerance of bulb scales of lily cultivars: effects of freezing and storage duration and partial dehydration

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Abstract

Effects of freezing duration, previous storage duration of bulbs at -2 □C, and partial dehydration of scales on freezing tolerance of lily (*Lilium* hybrids L.) scales were studied for a series of cultivars. Freezing tolerance of scales was estimated by measuring ion leakage and recording scale bulblet regeneration. Both methods gave similar results. Freezing tolerance decreased with freezing duration. A longer previous storage duration of the bulbs at -2 □C tended to reduce freezing tolerance of the scales. Dehydration of the scales to 10-20 % loss of water content significantly increased freezing tolerance. Further dehydration to 30-40 % loss of water content did not further increase freezing tolerance. Nucleation temperatures, temperatures during crystallisation and melting temperatures of the scales were recorded for the cultivar 'Enchantment'. Nucleation occurred at higher temperatures after a longer previous storage duration of bulbs, indicating a reduced capacity to remain supercooled. The increased freezing tolerance of dehydrated lily scales could partly be explained by a decreased melting temperature of the scales. We conclude long term storage of lily bulbs at -2 □C to be safer after partial dehydration to 10-20% loss of the original water content.

Introduction

In order to maintain a vegetative lily germplasm collection, bulbs can be stored for two to three years in moist peat at -2 □C (Bonnier *et al.*, 1996). Increasing the maximum length of the storage period would make the maintenance of a lily collection more efficient. Although a temperature of -2 □C is generally used to store lily bulbs, sometimes injury of sprouts occurs (Beattie and White, 1993).

Freezing tolerance can be increased by cold-acclimatization, by abscisic acid, partial dehydration, or low atmospheric pressure (Halloy and Gonzales, 1993; Lång *et al.*, 1994; Robertson *et al.*, 1994; Mantyla *et al.*, 1995). For storing a genetically diverse vegetative germplasm collection it is important to know the variation in freezing tolerance among genotypes. For long term storage, it is also important to know whether freezing tolerance is constant during storage. If freezing tolerance declines with storage duration, freezing injury can occur during prolonged storage.

Differences in freezing tolerance between plants can be based on differences in capacity to tolerate the formation of extracellular ice, on differences in osmotic potential of plant tissues and on differences in capacity to retain a supercooled state during freezing (Sutcliffe, 1977; Lipp *et al.*, 1994). The formation of intracellular ice is lethal to the cells (Sutcliffe, 1977; Levitt, 1980), unless special freezing techniques are used for cryopreservation (Withers, 1991).

The freezing of a cell suspension is described by Steponkus (1984). During cooling both the cells and the suspending medium initially supercool. Subsequently ice nucleation occurs at a temperature dependent on the freezing point of the medium and the presence of ice-nucleating agens. Ice formation will continue until the chemical potential of water in the unfrozen portion is in equilibrium with that of the ice. The intracellular solution must also come into equilibrium with the extracellular ice. Equilibrium is achieved either by intracellular ice formation or cell dehydration, dependent on the cooling rate and the stability of the plasma membrane.

The aim of our experiments was to determine freezing tolerance of different lily genotypes, and the way this tolerance is influenced by freezing duration, previous storage duration of bulbs, and partial dehydration of scales. Nucleation temperature, maximum temperature during freezing and melting temperature of scales were determined, with the aim

to link freezing tolerance of lily scales to supercooling capacity, osmotic potential, or capacity to tolerate ice formation.

Material and methods

Lily (*Lilium* hybrids L.) bulbs of 'Avignon', 'Enchantment', 'Gelria', 'Mont Blanc' and 'Star Gazer' with a circumference between 12 and 16 cm were obtained in autumn of 1992 and 1993 from commercial stocks. The bulbs were stored in moist peat at -2 □C. At the start of each experiment, bulbs were defrosted at 5 □C for three days. Then, the scales were removed and white scales were selected for uniformity. Three experiments were performed.

First experiment

Eight bulbs per genotype were used of 'Avignon', 'Gelria', 'Mont Blanc' and 'Star Gazer', stored for 0.7 years. Freezing tolerance was determined by exposing three scales per genotype to -2, -4, -5, -6, and -8 □C for 24 h and by exposing six scales per genotype to -2, -3, -4, -5, and -6 □C for 144 h in a cooled incubator (Sanyo MIR-552, fluctuation ± 1 □C).

Second experiment

Freezing tolerance of 'Gelria' scales from bulbs stored for 0.3 and 1.3 years (25 bulbs each) was estimated. Half of the scales from the bulbs stored for 0.3 years were air-dried at 17 □C under ventilation, until the loss of water content was between 10 and 20 %. The original water content of the scales (after storage, before drying) was 1.73 ± 0.16 $g.g^{-1}$ dry weight. The other scales were stored at 0 □C, until freezing tolerance was measured. Six scales per treatment were exposed to -2, -4, -6, -8, and -10 □C for 24 h to determine freezing tolerance.

Third experiment

Freezing tolerance of 'Enchantment' scales from bulbs stored for 0.5, 1.5 and 3.5 years (50 bulbs each) at -2 □C was estimated. Scales were air-dried at 17 □C under ventilation into three classes of water content loss: 0-5 %, 10-20 %, and 30-40 %. The original water content of the scales was 1.42 ± 0.08, 1.75 ± 0.24 and 1.80 ± 0.14 $g.g^{-1}$ dry weight for scales from 0.5, 1.5 and 3.5 years stored bulbs respectively. Six scales per treatment were exposed to -2, -4, -6, -8, -10, and -20 □C to determine freezing tolerance. Also, three 'Enchantment'

scales per treatment were used to measure the temperatures in the scales at the start of crystallisation (nucleation temperature), during crystallisation and during melting, using a copper/constantan thermocouple and a recorder.

Determination of freezing tolerance

After exposure to freezing temperatures scales were defrosted at 20 □C for 1 h and ion leakage tests were performed as described earlier (Bonnier *et al.*, 1994). Each scale was rinsed in distilled water and placed in 100 ml of distilled water at 20 □C. After 1.5 h, the conductivity of the external medium was measured using a digital conductivity meter (Philips PW9526 with electrode PW9514/60). The leakage from each scale was corrected for the initial conductivity of the distilled water (1.85 $TS.cm^{-1}$) and the initial (before drying) fresh weight of the scale. Freezing tolerance (FTcon) was estimated from the inflection point of a Gompertz-curve fitted by non-linear regression of electrical conductivity values on freezing temperatures. A Gompertz-curve is a sigmoidal curve, where the start of the increase is more abrupt than the end of the increase (Payne *et al.*, 1993). After measuring ion leakage, the scales were planted in moistened vermiculite and incubated at 25 □C for 8 weeks. Then, the percentage of the scales that had formed scale bulblets was determined as a measure of survival. Survival of the scales was regressed on freezing temperature using a generalized linear model for proportions (Payne *et al.*, 1993). Freezing tolerance was determined by the LT50, the temperature that is lethal to 50 % of the tested material (Dallaire *et al.*, 1994; Maier *et al.*, 1994; Mantyla *et al.*, 1995). The fitting of scale survival (Fig. 7.1A) and ion leakage (Fig. 7.1B) after exposure to freezing is presented for the three dehydration classes of scales of 0.5 years stored 'Enchantment' bulbs. The fitting of the curves for the other treatments was similar (results not shown).

Crystallisation in the scales

Scales were placed in the cooled incubator and the temperature was decreased from 10 □C to -12 □C in approximately 30 min. The nucleation temperature and the temperature during crystallisation were determined per scale as described by Levitt (1980). The start of ice crystallisation was observed by a sudden increase in temperature, rising from the nucleation temperature to the maximum temperature during freezing during crystallisation (Fig. 7.2). An accurate estimate of melting temperatures during a constant rise of temperature was not

possible, because a clear halt of the temperature rise inside the scales during the melting was not observed.

Figure 7.1 The fitting of scale survival (**A**) and ion leakage (**B**) of lily scales 'Enchantment' after 6 days exposure to freezing. Before freezing, scales were air-dried and distributed in three classes of water content loss: 0-5% (—✍—), 10-20% (--💻--), and 30-40% (¨❑¨). Scales were taken from bulbs stored for 0.5 years in moist peat at -2 □C. Survival was determined by the percentage of scales that regenerated scale bulblets after freezing, and fitted by a

generalized linear model for proportions. Ion leakage was measured by the electrical conductivity of external solution after 1.5 h leakage of lily scales and fitted by a Gompertz curve. Each point represents the mean of 6 scales.

The melting temperature was estimated by increasing the temperature of frozen scales stepwise with 1 □C every 6 h, starting at -4.5 □C. After each temperature step, a 1.5 h temperature decrease to -12 □C was given. If scales had thawed, an exotherm was observed during this cooling (Fig. 7.2). The proportion of thawed scales per temperature was regressed on the temperature using a generalized linear model for proportions, giving estimates for the temperature at which half of the scales had thawed (TT50). Significances ($P \leq 0.05$) of differences between storage duration of bulbs and dehydration classes of scales were calculated for the nucleation temperature and the maximum temperature during freezing by using student t-tests. Significances ($P \leq 0.05$) between melting temperatures (TT50's) were calculated by using the rank sum test of Mann-Whitney for two independent samples (Snedecor and Cochran, 1980).

Figure 7.2 Determination of the nucleation temperature (**A**), the maximum temperature during freezing (**B**), and the melting temperature range (**C**) of lily scales. The temperature was regulated by a cooled incubator and was measured in the scales by a copper/constantan thermocouple. Further method description: see page 61.

Results

Determination of freezing tolerance

Scales exposed to 24 h freezing survived lower temperatures than those exposed to 144 h freezing, the difference ranging from 0.7 □C ('Mont Blanc') to 2.0 □C ('Avignon') for bulbs previously stored for 0.7 year (Table 7.1). This means that freezing tolerance decreased with freezing duration. Longer duration than 144 h will probably further decrease freezing tolerance. Freezing tolerance was also genotype dependent. 'Mont Blanc' was relatively tolerant to freezing, whereas 'Gelria' was more sensitive (Table 7.1). The LT50 of non-dehydrated scales from 0.7 y stored bulbs, varied between -4.9 and -7.0 □C after 24h freezing and between -4.1 and -6.3 after 144 h freezing (Table 7.1).

Freezing tolerance of non-dehydrated scales tended to decrease with longer previous storage duration of bulbs, but the results were not significant. The LT50 after 24 h freezing exposure of non-dehydrated 'Gelria' scales stored for 1.3 years was higher than the LT50 of 0.3-years-stored and 0.7-years-stored 'Gelria' scales (Table 7.1). In addition, the LT50 after 144 h of freezing exposure of non-dehydrated scales from 1.5 years stored bulbs of 'Enchantment' was higher than that of non-dehydrated scales of 0.5 years stored 'Enchantment' bulbs (Table 7.1). Unfortunately, freezing tolerance of 3.5 years stored 'Enchantment' bulbs could not be determined, because survival was less than 100 %, and ion leakage was increased of scales that were not exposed to freezing. For dehydrated scales, no indication was found for a decrease in freezing tolerance with previous storage duration of bulbs.

Dehydration of scales increased freezing tolerance significantly for all tested bulbs. LT50 of scales from 0.3-years-stored 'Gelria' bulbs decreased 1.2 □C after 10-20 % loss of water content and LT50 of scales from 0.5 and 1.5-years-stored 'Enchantment' bulbs decreased 2.0 and 1.5 □C, respectively, after 10-20 % loss of water content (Table 7.1). Dehydration of 'Enchantment' scales to 30-40 % loss of water content did not further increase freezing tolerance (Table 7.1). For dehydrated scales, there was no indication of a decrease in freezing tolerance with previous storage duration of bulbs. Dehydration of scales caused increased ion leakage at -2 □C, without influence on survival (Fig. 7.1A,B).

Freezing tolerance estimated by survival of the scales (LT50) and freezing tolerance estimated by ion leakage of the scales (FTcon) gave similar results (FTcon = 0.97 * LT50 - 0.16) and were highly correlated ($r^2 = 0.90$).

Table 7.1 Freezing tolerance (LT50 + 95 % confidence interval) of scales from lily bulbs stored for different times at -2 □C, dehydrated and distributed in three classes of water content loss, exposed for 24 h and 144 h to freezing.

Cultivar	Storage duration (year)	Year of harvest	% loss of initial water content	Estimated LT50 (□C)	95 % confidence interval
Freezing exposure = 24 h					
Gelria	1.3	1992	0-5	-4.2	(-3.4;-5.1)
Gelria	0.7	1992	0-5	-4.9	(-4.4;-5.4)
Gelria	0.3	1993	0-5	-5.8	(-4.9;-6.6)
Gelria	0.3	1993	10-20	-7.0	(-6.4;-7.6)
Avignon	0.7	1992	0-5	-6.1	(-5.6;-6.8)
Star Gazer	0.7	1992	0-5	-6.1	(-5.6;-6.8)
Mont Blanc	0.7	1992	0-5	-7.0	(-6.4;-7.6)
Freezing exposure = 144 h					
Gelria	0.7	1992	0-5	-4.1	(-3.7;-4.6)
Avignon	0.7	1992	0-5	-4.1	(-3.7;-4.6)
Star Gazer	0.7	1992	0-5	-5.1	(-4.7;-5.4)
Mont Blanc	0.7	1992	0-5	-6.3	(-6.0;-7.1)
Enchantment	1.5	1992	0-5	-5.3	(-4.8;-5.8)
Enchantment	0.5	1993	0-5	-5.8	(-4.9;-6.5)
Enchantment	0.5	1993	10-20	-7.8	(-7.0;-8.5)
Enchantment	0.5	1993	30-40	-8.0	(-7.1;-8.9)
Enchantment	1.5	1992	10-20	-8.4	(-8.1;-9.2)
Enchantment	1.5	1992	30-40	-8.4	(-8.1;-9.2)

Crystallisation in the scales

The nucleation temperature was higher in scales from 3.5-years-stored 'Enchantment' bulbs than in scales from 0.5- and 1.5-years-stored bulbs, but duration of previous storage had no influence on the maximum temperature during freezing and the melting temperature of the scales (Fig. 7.3A). These results indicate a reduced capacity of scales from 3.5-years-stored bulbs to remain supercooled.

Dehydration of scales did not have a significant effect on the nucleation temperature (Fig. 7.3B). The maximum temperature during freezing was not significantly changed after 10-20 % loss of the water content, but dehydration to 30-40 % loss of the original water content significantly decreased this temperature. Dehydration of scales decreased their melting temperatures significantly (Fig. 7.3B).

Figure 7.3 Nucleation temperature (Tn), maximum temperature during freezing (Tf), and melting temperature (TT50) of lily scales 'Enchantment' exposed to a temperature declining from 10 □C to -12 □C in about 30 minutes. (**A**): Scales were taken from bulbs stored previously for 0.5, 1.5 and 3.5 years at -2 □C; (**B**): Scales were air-dried and distributed into three classes of water content loss (all the three storage durations). Different minor characters indicate significant differences ($P \leq 0.05$) between treatments. Nine to twelve scales were used per treatment.

Discussion

Significant differences in freezing tolerance were found between cultivars. The cultivars with the lowest freezing tolerance determine the minimum storage temperature of a germplasm collection. These cultivars were 'Avignon' and 'Gelria', which both had an LT50 of -4.1 □C after 144 h freezing exposure. However, freezing damage occurs probably at higher temperatures during prolonged storage, because freezing tolerance declined with freezing duration, and injury of sprouts is sometimes observed during prolonged storage of lily bulbs at -2 □C (Beattie and White, 1993).

Freezing tolerance of non-dehydrated lily scales tended to decline with previous storage duration of bulbs at -2 □C. Storage of bulbs only increased the nucleation temperature and had no effect on the maximum temperature during freezing and the melting temperature of the scales. With a constant temperature decline, a smaller difference in nucleation temperature and maximum temperature during freezing indicates a reduced capacity of scales to remain supercooled. Retaining a state of supercooling during freezing is one of the ways plants can avoid freezing damage by ice crystallisation (Lipp *et al.*, 1994).

Dehydration to 10-20 % loss of water content increased freezing tolerance of 'Gelria' and 'Enchantment'. These results are in accordance with earlier reports on other plants (Lång *et al.*, 1994; Pearson and Davison, 1994; Silim and Lavender, 1994; Mantyla *et al.*, 1995). Further dehydration to 30-40 % loss of water content did not significantly further increase freezing tolerance.

The plasmalemma is a primary site of freezing injury leading to cell leakage (Steponkus, 1984; Samygin, 1994). Therefore, ion leakage can be used to determine freezing injury (Bonnier *et al.*, 1992; Bigras and Calme, 1994; Maier *et al.*, 1994). Freezing tolerance can be estimated by the inflection point of a sigmoidal curve fitted by non-linear regression of ion leakage on temperature (Fry *et al.*, 1991; Maier *et al.*, 1994). We estimated freezing tolerance (FTcon) by the inflection point of a Gompertz-curve fitted by non-linear regression (Payne *et al.*, 1993) of the natural logarithm of electrical conductivity of external solution on temperature. FTcon and LT50 were nearly equal and highly correlated. FTcon was measured 8 weeks earlier than LT50.

Freezing injury in plants is a complex phenomenon that has been extensively studied (Levitt, 1956, 1980; Ristic and Ashworth, 1993; Samygin, 1994; Steponkus, 1984; Sutcliffe, 1977). Freezing of tissue results in the formation of extracellular or intracellular ice, dependent on cooling rate and the presence of ice-nucleating agents. At low cooling rates, ice formation is extracellular and water efflux from cells concentrates the intracellular solutes, decreasing the intracellular freezing point (Steponkus, 1984). The formation of extracellular ice can lead to injury, but not necessarily. The injury is dependent on plant species, plant tissue, cooling rate, length of freezing period, and thawing conditions (Levitt, 1980). At high cooling rates, water efflux from cells is not sufficiently rapid and intracellular ice formation may follow supercooling of the intracellular solution (Steponkus, 1984). In our experiment the cooling rate was 44 □C per h. We could not distinguish between extracellular or intracellular ice formation in the lily scales.

Though freezing tolerance of 10-20 % dehydrated 'Enchantment' scales was increased by 2.0 □C, significant changes in nucleation temperature were not found, and maximum temperature during freezing was only decreased significantly after 30-40 % dehydration (Fig. 7.3B). The melting temperature of 10-20 % dehydrated 'Enchantment' scales was only slightly decreased. This means, that the increase in freezing tolerance is not fully explained by the decrease in melting temperature.

During storage at a supercooled state, crystallisation can start at any moment. Crystallisation is probably lethal to the scales and scales seem to lose their capacity to retain a supercooled state during long term storage. Therefore, lily bulbs should not be stored for a prolonged period at a supercooled state. We conclude, that the risk of freezing injury of lily scales during prolonged storage at -2 □C can be decreased by dehydrating the scales to 10-20 % loss of the original water content.

Chapter 8

Viability loss and oxidative stress in lily bulbs during long-term cold storage

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Abstract

The regeneration ability of bulb scales of the Asiatic hybrid lily (*Lilium* hybrids L.) 'Enchantment' was monitored for bulbs stored for 0 to 5 years at -2 □C in moist peat. Regeneration ability decreased after more than 1 year of storage and was completely lost after 5 years. Possible involvement of oxidative stress in the loss of regeneration capacity was tested. In this study we used white (i.e. with no visual damage) scales to test whether breakdown of membranes by oxidative stress was an early event in this storage-induced viability loss of lily bulbs. The content of reduced glutathione, which was the main non-protein thiol in the bulb scales, remained nearly constant, while that of oxidized glutathione was slightly increased after 3.3 years of storage. Significant changes in the content of phospholipids, neutral lipids and free fatty acids were not detected during storage. The degree of unsaturation of fatty acids in phospholipids increased during the first 3 years of storage. Ion leakage of bulb scales was not increased in bulbs that were stored for less than 4 years. No indication was found that oxidative stress is a major factor associated with the loss of regeneration capacity of lily bulbs during cold storage.

Introduction

Lily bulbs can be propagated by regeneration of scale bulblets on detached scales from bulbs. Scale bulblets are formed adventitiously at the bottom of the detached lily scales during storage at 25 □C in vermiculite or in moist peat. These scale bulblets can be grown to larger bulbs after breaking dormancy by cold treatment (Beattie and White, 1993). For maintenance of lily vegetative germplasm collections and for year-round cut flower production, lily bulbs are usually stored at -2 □C in moist peat (Beattie and White, 1993; Bonnier and Van Tuyl, 1996). During this storage, however, bulbs lose their viability, which is manifested by a decreased potency of scales to regenerate scale bulblets and is associated with increased ion leakage (Bonnier and Van Tuyl, 1996; Bonnier *et al.*, 1994). The physiological and biochemical processes causing this viability loss during storage are not known. Insight into these processes would be beneficial for the development of more successful storage techniques.

Free radical-induced oxidative stress is thought to be a major process in senescence and ageing of plant tissues (Benson, 1990; Leshem, 1988; Mckersie *et al.*, 1988; Paliyath and Droillard, 1992). Oxidative stress has been shown to take place during storage of seeds (De Vos *et al.*, 1994; Pukacka and Kuiper, 1988; Salama and Pearce, 1993; Stalleart and Geuns, 1994), dry pollen (Van Bilsen *et al.*, 1994a; Van Bilsen *et al.*, 1994b), potato tubers (Kumar and Knowles, 1993) and several other agricultural products (Deschene *et al.*, 1991; Lester and Stein, 1993). Oxidative stress is also involved in loss of viability of plants exposed to a variety of environmental stress situations, such as chilling (Hariyadi and Parkin, 1991), freezing (Steponkus, 1984; Okuda *et al.*, 1994), exposure to copper (De Vos *et al.*, 1992), exposure to environmental pollution (Benson, 1990) and pathogenic disease (Bradley *et al.*, 1992). There are various defence mechanisms in higher plants, both enzymatic and non-enzymatic, which prevent damage induced by free radicals (Benson, 1990; Leshem, 1988). One of these mechanisms involves the tripeptide glutathione (GSH). Upon oxidative stress, GSH can be oxidized forming GSSG. GSSG is converted back to GSH by glutathione reductase (GR) and NADPH (Benson, 1990; Leshem, 1988).

Glutathione depletion and oxidation, decrease in phospholipid content, increase in free fatty acid content, and increase in saturation level of fatty acids, are considered to be associated with oxidative stress (Benson, 1990; Leshem, 1988; Mckersie *et al.*, 1988; De Vos

et al., 1994; Van Bilsen *et al.*, 1994a; Van Bilsen *et al.*, 1994b). Oxidative stress often results in increased ion leakage, because it leads to membrane breakdown (Benson, 1990; Leshem, 1988).

The goal of this study was to ascertain whether oxidative stress is involved in the loss of viability of lily bulb scales during storage at -2 □C in moist peat. We therefore analyzed regeneration and ion leakage of scales as indicators of viability and membrane damage, respectively. As indicators of oxidative stress, we analyzed glutathione depletion and oxidation, decrease in phospholipid content, and increase in free fatty acid content and in saturation level of fatty acids.

Material and methods

Plant material

Lily (*Lilium* hybrids L.) bulbs of the Asiatic hybrid 'Enchantment' with a circumference between 12 and 16 cm were obtained from commercial stocks every year in autumn from 1989 to 1994. The bulbs were stored in moist peat at -2 □C. The experiments were carried out in 1994, when the storage periods ranged from 0 to 5 years.

Viability of bulbs

For each test, five bulbs per age were defrosted at 5 □C for three days and all scales (varying from 20 to 60 scales per bulb) were removed and sampled per bulb. Scales were disinfected in a solution containing 1% captan and 0.2% prochloraz for 10 min, dried for 4 h, and then planted in peat and placed in a greenhouse at 20 □C. After 8 weeks the proportion of regenerative scales per bulb was recorded by counting the scales that regenerated one or more bulblets. Bulbs of all 5 ages were tested at the end of 1994. Bulbs stored for 2, 3, and 4 years were also tested at the end of 1993.

Ion leakage of white scales

Ion leakage tests were performed as described previously (Bonnier *et al.*, 1994). Briefly, five bulbs per age were defrosted for three days at 5 □C and scaled. From every bulb one white scale was taken. Scales were washed with water and stored over-night in a polyethylene bag at 18-20 □C to give fresh wounds time to heal. Each scale was then

washed in distilled water and put in 100 ml of distilled water at 20 □C. After 1.5 h, the conductivity of the external medium was measured using a digital conductivity meter (Philips PW9526 with electrode PW9514/60). The leakage from each scale was corrected for the initial conductivity of the distilled water (1.85 $TS.cm^{-1}$) and expressed on a fresh weight basis. Ion leakage tests were not performed with scales older than 4 years, because they were (partly) deteriorated and had turned brown.

Indices of oxidative stress

Extraction and assays of non-protein thiols. Three bulbs per age were taken out of the -2 □C storage and scaled in ice-cold water. Per bulb, the scales were pooled excluding brown scales and brown parts of scales. The collected scales were frozen in liquid N_2 and powdered in an IKA analysis mill, model A10 (Janke and Kunkel KG, IKA Werk, Staufen in Breisgau, Germany). The powder was freeze-dried and stored at -80 □C until use. Non-protein thiols were extracted and assayed according to De Vos *et al.* (1992). Approximately 20 mg of freeze-dried material was homogenized for 3 x 15 s in 0.5 ml 5 % (w/v) SSA containing 6.3 mM DTPA at 0 □C, using an Ultraturrax homogenizer. An additional 0.5 ml of SSA/DTPA solution was added, and the material was homogenized once more for 15 s. The homogenate was placed on ice for 10 min for protein precipitation, centrifuged at 10.000 g for 10 min, and the clear supernatants were collected and kept on ice. The level of total acid-soluble SH compounds was determined with Ellman's reagent, while both GSH and GSSG were assayed by the enzymatic GSSG-recycling method (Anderson, 1985), as modified by De Vos *et al.* (1992). The glutathione oxidation state is expressed as the proportion of GSSG of total glutathione (GSSG expressed as GSH equivalents).

Extraction and assays of phospholipids, neutral lipids and free fatty acids. Analyses of phospholipids, neutral lipids and free fatty acids were modified from earlier described methods (Hoekstra and Van Roekel, 1988). Five hundred mg samples of ground, freeze-dried material (see above) were extracted with 50 ml of chloroform:methanol (1:1). As the internal standards 1.5 mg diheptadecanoyl phosphatidylcholine, 0.5 mg triheptadecanoin and 0.5 mg heptadecanoic acid were added. The mixture was subjected to 5 min of mild ultrasonic treatment. The homogenates were centrifuged for 5 min at 1000 x g in glass tubes. The

supernatants were collected, mixed with 12 ml of a 0.9 % NaCl solution and again centrifuged to aid phase separation. The chloroform layer was collected and dried by passage over a column of anhydrous Na_2SO_4. After vacuum evaporation the material was dissolved in 1 ml chloroform. Total lipid extracts were separated into neutral and polar lipid fractions by passing them over a SEP-PAK silica cartridge (Waters Associates, Milford, Mass., USA, catalog no. 51900). Neutral lipids and free fatty acids were eluted from the column with 30 ml chloroform. Then the column was washed with acetone. Subsequently, phospholipids were eluted with 30 ml methanol. After vacuum evaporation, part of the fractions were mixed with 3 ml of 0.3 N KOH in methanol. Transmethylation was carried out for 15 min at 70 □C with vigorous shaking. After cooling on ice and adding 1 ml saturated NaCl-solution and 1.5 ml hexane, the methylated fatty acids were phase-partitioned to hexane (centrifugation) and dried over anhydrous Na_2SO_4 before GC analysis. Fatty acid methylesters were analyzed on a Shimadzu GC8A GC, equipped with a 30 m JandW DB225 megabore column (JandW Scientific, Folsom Calif., USA) and a flame ionisation detector, coupled to a Spectra Physics SP4100 integrator. Identification was by comparing with standards (Sigma, St Louis, MO, USA). Contents were calculated on the basis of peak surface areas of the added internal standards.

The neutral lipid fraction was also separated on Silica Gel-G TLC plates using hexane:diethylether:acetic acid (80:20:1, v/v/v) as the developing solvent. After the plates were sprayed with 0.1% 8-anilino-1-naphtalene sulfonic acid in methanol and inspected with UV-light the free fatty acid band was scraped off. After extraction, the free fatty acids were methylated using freshly prepared diazomethane in diethylether and analyzed on the GC as described above.

Statistical analyses

The regeneration of bulblets on scales was regressed on storage duration using a generalized linear model for proportions (Payne *et al.*, 1993). Electrical conductivity values were log transformed, to obtain a normal distribution. Student t-tests were used to calculate LSD-values for differences between treatments (significant at I = 0.05).

Results

Viability of bulbs

More than 92 % of the scales of bulbs stored for 1 year or less regenerated scale bulblets (Fig. 8.1). The proportion of scale regeneration of bulbs stored from 2 to 4 years was lower and more variable for the different bulb samples. The ability to regenerate was completely lost for the sample of bulbs stored for 5 years (Fig. 8.1). Scales of old bulbs had often turned completely or partly brown, and deteriorated.

Figure 8.1 Effect of storage at -2 □C in moist peat on the ability of lily bulb scales of 'Enchantment' to regenerate scale bulblets. The scales with and without regeneration were counted (✂ = harvest 1994, 💻 = harvest 1993, ❑ = harvest 1992, 🚗 = harvest 1991, ⌛ = harvest 1990, ◑ = harvest 1989). Data represent means ± SD of five bulbs (20 to 60 scales each).

Ion leakage of white scales

The plasma membrane integrity of bulb cells was tested by measuring ion leakage from white bulb scales. Leakage of scales stored for 3.5 years or less was not significantly higher than that of fresh scales (Fig. 8.2). However, leakage from scales of bulbs stored for 4

years was significantly increased. Thus, the plasma membrane integrity of the cells in the white scales was not significantly affected until 4 years of storage.

Figure 8.2 Effect of storage at -2 □C in moist peat on ion leakage of lily bulb scales of 'Enchantment'. Ion leakage was determined by incubating one white (no visual damage) scale per bulb in 100 ml of distilled water for 1.5 h at 20 □C, and measuring the electrical conductivity of the external solution before and after incubation (✂ = harvest 1994, 💻 = harvest 1993, ❑ = harvest 1992, 📎 = harvest 1991, ⌛ = harvest 1990). Data represent means ± SD of five bulbs.

Indices of oxidative stress

Content and oxidation state of glutathione. GSH represented approximately 95 % of total acid-soluble SH compounds in all extracts. The content of GSH was always between 1.3 and 2.2 Tmole.g^{-1} dry weight, except for the storage duration of 4.3 years (Fig. 8.3A). After more than 3.5 years of storage, the GSSG content was slightly increased (Fig. 8.3A), while the glutathione oxidation state became only more variable (Fig. 8.3B). Yet, for the bulbs harvested in both 1989 and 1990 the oxidation of glutathione was increased during the last 0.6 years of storage (Fig. 8.3B).

Figure 8.3 Effect of storage at -2 □C in moist peat on content of reduced glutathione (GSH) and oxidized glutathione (GSSG) (**A**); and oxidation state of glutathione (**B**) in lily bulb scales of 'Enchantment' (💻 = harvest 1993, ❑ = harvest 1992, 🥽 = harvest 1991, ⌛ = harvest 1990, ◑ = harvest 1989). Data represent means ± SD of three bulbs (20 to 60 scales each).

Figure 8.4 Effect of storage at -2 □C in moist peat on: content of phospholipids (**A**), content of neutral lipids (**B**), content of free fatty acids (**C**) and the degree of unsaturation of fatty acids in phospholipids [expressed as mean number of double bonds per fatty acid] (**D**) in lily bulb scales of 'Enchantment' (□ = harvest 1993, ❑ = harvest 1992, □ = harvest 1991, □ = harvest 1990, □ = harvest 1989). Data represent means ± SD of three bulbs (20 to 60 scales each).

Changes in phospholipids and fatty acids. Although some significant differences in contents of phospholipids (Fig. 8.4A) and neutral lipids (Fig. 8.4B) were observed, a clear relation with storage duration was not detected. Significant differences in free fatty acid content were not found during the five years of storage (Fig. 8.4C). Also a decrease in the degree of unsaturation of the fatty acids (a measure of lipid peroxidation) was not observed during the 5 years of storage (Fig. 8.4D). On the contrary, the number of double bonds per fatty acid in phospholipids increased significantly during the first 3 years of storage and remained constant thereafter at this elevated level (Fig. 8.4D). The increase in the number of double bonds per fatty acid was mainly caused by an increase in linolenic acid content (18:3) and a decrease in palmitic acid (16:0) and linoleic acid (18:2) (Fig. 8.5). Also on the basis of the dry weight, the content of linolenic acid increased from 0.04% to 0.08%. For palmitic acid the contents decreased from 0.12% to 0.07% and for linoleic acid from 0.32% to 0.20. The contents of the other fatty acids, stearic acid (18:0) and oleic acid (18:1), were less than 3.2 mol percent, without significant changes (data not shown).

Figure 8.5 Effect of storage duration at -2 □C in moist peat on contents of 18:2 and 18:3 fatty acids in mol percent of total fatty acids analyzed in phospholipids in lily bulb scales of 'Enchantment' (💻 = harvest 1993, ❑ = harvest 1992, 🚗 = harvest 1991, ⌛ = harvest 1990, ◑ = harvest 1989). Data represent means ± SD of three bulbs (20 to 60 scales each).

Discussion

It was possible to regenerate scales from all bulbs tested during the first three years of storage. These results are in agreement with earlier results (Bonnier *et al.*, 1994), showing that lily bulbs of Asiatic hybrids can be stored in moist peat at -2 □C for at least three years. Infection by *Penicillium* was sometimes observed on brown scales. This infection could be the primary cause of the browning of the scales, but could also have followed damage of the scales during storage. The proportion of scale regeneration was determined on all scales per bulb, including brown scales and scales with brown areas. To test whether oxidative processes were an early event prior to scale browning, the assays of indices of oxidative stress were carried out on clean white scales or white parts of scales directly after storage at -2 □C.

Ion leakage from white scales gave no indication that the plasma membrane integrity of the cells involved was affected until 4 years of storage. The significantly increased ion leakage at a storage duration of 4 years probably indicated membrane damage, because it was accompanied by a low proportion of scale regeneration (Fig. 8.1 and 2, harvest 1990). Leakage measurements were made on tissue that had been thawed for three days. During this thawing time, initially damaged cells could have been repaired, or membrane damage could have resulted from peroxidation of membrane lipids (Benson, 1990; Leshem, 1988) as a secondary consequence from another kind of primary injury to the cells. Membrane damage could also have resulted from other physical changes at the membrane level, e.g. phase transitions (Van Bilsen *et al.*, 1994a) or damage by ice crystals (Steponkus, 1984).

Thiols are among the cellular compounds that are initially affected upon oxidative stress, due to the susceptibility of their SH groups (De Vos *et al.*, 1994). The non-protein thiol fraction in lily scales consisted for 95 % of GSH. Contents of GSH and GSSG, and the glutathione oxidation state did not change significantly over the first 3.3 years of storage, giving no indication of the occurrence of oxidative stress. However, the increase in glutathione oxidation of bulbs stored for 3.9 and 4.9 years, in comparison with bulbs of the same harvests stored for 3.3 and 4.3 years, respectively, might be an indication of oxidative stress in these bulbs. We have no clear explanation for the high content of GSH at a storage duration of 4.3 years (Fig. 8.3A). The extraction and measurements of this sample were repeated, but the same high value of GSH-content was found. Possibly, the production of GSH in these scales might be a reaction of the cells to the first occurrence of oxidative stress.

During the 5 years of storage, no decrease in the content of phospholipids and neutral lipids (Fig. 8.4A,B) and no increase in content of free fatty acids (Fig. 8.4C) were found. The unsaturation level of phospholipid fatty acids increased during storage (Fig. 4D). This was unexpected because fatty acid peroxidation would have led to a decrease in unsaturation level. Therefore, the contents of phospholipids and fatty acids, and the unsaturation level gave no indication for the occurrence of membrane breakdown by oxidative stress. The increase in unsaturation of phospholipid fatty acids during storage at -2 □C might be regarded as an adaptation to low temperature, increasing the flexibility of the membranes and decreasing the liquid crystalline-to-gel phase transition temperature (Hoekstra *et al.*, 1992). Increase in unsaturation of fatty acids is generally reported as a reaction to exposure to low temperatures (McKersie *et al.*, 1988; Bulder *et al.*, 1991). An increase in the ratio of the 18:3/18:2 fatty acids was reported for potato tubers after 40 weeks of storage at 3 □C (Spychalla and Desborough, 1990) and for tulip scales within 20 weeks of storage at both 5 □C and 17 □C (Walch and Van Hasselt, 1991). Here, it is shown that the increase in the degree of unsaturation continues over 3 years of storage at -2 □C.

We did not succeed in determining the level of lipid peroxidation products by measuring 2-thiobarbituric acid-reactive material (De Vos *et al.*, 1989), because we could not distinguish a peak at 532 nm, due to a high absorbance at 480 nm (results not shown).

Although free radical-induced oxidative stress is thought to be a major process in senescence and ageing of plant tissues (Benson, 1990; Leshem, 1988; Mckersie *et al.*, 1988; Paliyath and Droillard, 1992), we found no indication for oxidative stress in white scales or white parts of the scales of lily 'Enchantment' bulbs until after a storage duration of 3.9 years. Our results do not provide conclusive evidence for the absence of oxidative stress in lily scales because oxidative damage could have occurred in only a few cells of the scales, which are involved in the adventitious regeneration. The methods used in this study to assay oxidative damage are not sensitive enough to detect such small contributions of oxidative stress. A few damaged cells could give access to micro-organisms causing the reported browning of the scales.

Chapter 9

General discussion

Introduction

The preservation of a broad genetic variation in germplasm collections is important for breeding crops, research, and combatting genetic erosion (Wehner, 1988). Lily germplasm must be preserved vegetatively as clones, because the genotypes are unique and heterozygous. Using seeds would affect these unique genetic combinations. Collections of clonal lines are usually maintained in the field (Towill, 1988), leading to high labour and other costs and risking losses caused by diseases. Hence, storage of clonal material for at least two years would be very useful. Therefore, in 1991 a study was started at CPRO-DLO aiming at the development of methods for long term storage of lily germplasm.
The research on long term storage of clonal material of lily comprised several aspects. Methods to measure viability of lily scales were studied (Chapter 2,3), storage methods for bulbs, scale bulblets, and *in vitro* bulblets were developed (Chapter 4,5,6), freezing tolerance of lily scales was measured and increased (Chapter 7), and the role of oxidative stress as a factor limiting maximum storage duration of bulbs was analyzed (Chapter 8). In this chapter, the results obtained and perspectives for lily germplasm preservation will be discussed.

Viability of lily scales and scale bulblets

In order to develop optimal storage methods, it was necessary to be able to measure the effects of different storage conditions on the viability of the lily material. Most useful would be a fast and easy test for viability. Viability of bulbs was measured on the formation of scale bulblets on detached scales. The proportion of regenerative scales decreased with storage duration at -2 □C to zero after 5 years (Chapter 4). Viability declined faster in outer scales than in middle and inner scales (Chapter 3). Surprisingly, the weight of the bulblets

formed on inner scales after storage increased during 2.5 years of storage. This increase in weight was not accompanied by a decrease in the number of bulblets formed, except for the cultivar 'Avignon'. The increase in the production of scale bulblets with storage duration may be useful for the propagation of a lily collection. Viability of outer scales and some middle scales decreased during storage, as was detected by a lower percentage of regenerative scales, and per regenerative scale more and smaller scale bulblets were formed. This was also described by Matsuo and Arisumi (1978) for *L. longiflorum*.

Because the formation of scale bulblets on scales can only be detected after several weeks (Van Tuyl, 1983; Beattie and White, 1993), research was started to find a criterion for viability of lily scales that could be used in a earlier stage. Ion leakage was expected to be a useful criterion for viability of lily scales, because it is generally used to determine seed viability (Schmidt and Tracy, 1988; Tracy and Juvik, 1988), and various types of injury in leaves and fruits of various crops (McKersie *et al.*, 1981). Ion leakage from lily scales measured by the electrical conductivity of external solution appeared to increase with damage caused by frost, heat or dehydration (Chapter 2), and with viability loss during storage (Chapter 3). Therefore, ion leakage was used in further research (Chapter 4,7,8). Because outer scales lose viability earlier than inner scales (Chapter 3; Matsuo and Arisumi, 1978), maximum storage duration of bulbs is determined by the viability of the inner scales. Therefore, ion leakage from inner scales was used to estimate maximum storage duration of bulbs (Chapter 4).

Ion leakage from whole scale bulblets did not appear a useful criterion to measure viability of these bulblets (Chapter 5). During storage in a MA package at both -2 □C and 0 □C a decline of the sprouting proportion was often recorded without an increase in electrical conductivity. This difference might be explained by the fact that the sprouting proportion is determined by the health status of the meristemic cells, whereas ion leakage is mainly determined by the health status of the cells of the outer layers of the scales. When the meristem in a scale bulblet is damaged and the surrounding scales of the scale bulblet are still healthy, the scale bulblet will not sprout, but will show limited ion leakage. Therefore, sprouting of scale bulblets was the only useful criterion for viability of scale bulblets.

Freezing tolerance of lily bulb scales

In all preservation experiments, cold storage at -2 □C was used to minimize the metabolism of the stored germplasm. A lower temperature than -2 □C will further minimize this metabolism, and may increase maximum storage duration, but this is limited by the freezing tolerance of the germplasm. A temperature of -2 □C is generally used to store lily bulbs, although sometimes injury of sprouts occurs at this temperature (Beattie and White, 1993; Boontjes, 1983).

The genotypes with the lowest freezing tolerance determine the minimum storage temperature of a germplasm collection. In the lily collection studied, these genotypes were 'Avignon' and 'Gelria', with an LT50 (the temperature at which half of the scales did not survive) of -4.1 □C after a 144 h freezing exposure. However, LT50 is not easily translated to a minimum temperature for prolonged storage. LT50 was measured during a limited (24 h and 144 h) period of freezing and declined with exposure time. Moreover, LT50 estimates the temperature at which half of the scales do not survive. A fifty percent survival is not acceptable for germplasm storage. Therefore, the minimum storage temperature must be higher than the LT50 value. An accurate method to determine the minimum storage temperature for the lily collection is to monitor viability of 'Avignon' and 'Gelria' during prolonged storage at temperatures between -2 □C and -4.1 □C.

The freezing tolerance of lily scales tended to decline with the length of previous storage of bulbs at -2 □C. Storage of bulbs only increased the nucleation temperature and had no effect on the equilibrium freezing temperature and the melting temperature of the scales, indicating a reduced capacity of scales to remain supercooled (Lipp *et al.*, 1994). However, storage in a supercooled state should be avoided anyway, because crystallization may occur on small disturbances, inducing injury.

Dehydration of 10-20 % of original water content decreased the LT50 with 1.2 and 2.0 □C for 'Gelria' and 'Enchantment' scales, respectively (Chapter 7). These results are in accordance with earlier reports on other species (Lång *et al.*, 1994; Pearson and Davison, 1994; Silim and Lavender, 1994; Mantyla *et al.*, 1995). Dehydration of 30-40 % of original water content did not significantly further increase the freezing tolerance. Therefore, it was concluded that the risk of freezing injury of lily scales during prolonged storage at -2 □C can

be minimised by a previous dehydration of 10-20 % of the original water content of the scales (Chapter 7). The relation between freezing tolerance and partial dehydration is particularly important for storage of lily germplasm in MA packages (Chapter 5), because germplasm can be stored partly dehydrated in these packages.

Halloy and Gonzalez (1993) found an inverse relation between freezing tolerance of five plant species and atmospheric pressure. Therefore, research on the relation between freezing tolerance of lily germplasm and atmospheric pressure would give perspectives to find a method to increase the freezing tolerance of the lily germplasm. They suggested that the reduced oxygen level might have been one of the factors that increased freezing tolerance might have resulted from. Therefore, also research on the relation between partial oxygen pressure and freezing tolerance of lily germplasm would give perspectives to decrease the minimum storage temperature. Storage in MA packages is an easy way to store germplasm under reduced oxygen pressure (Chapter 5).

The perspectives to decrease the minimum temperature for prolonged storage of lily germplasm *in vitro* are not good. After several months, the media in some glass tubes froze at -2 □C, even when the freezing point of the media was depressed by addition of 9 % (w/v) sucrose (Chapter 6).

Viability loss and oxidative stress during storage of lily bulbs

Free radical-induced oxidative stress is thought to be a major factor in senescence and ageing of plant seeds and tissues (Benson, 1990; Kumar and Knowles, 1993; Leshem, 1988; McKersie *et al.*, 1988; Paliyath and Droillard, 1992; Sathiyamoorthy and Nakamura, 1995; Van Bilsen *et al.*, 1994b). Oxidative stress is also a factor in the loss of viability of plants exposed to environmental stress conditions, such as chilling (Hariyadi and Parkin, 1991), freezing (Steponkus, 1984), excess of copper (De Vos *et al.*, 1992), salt stress (Hernandez *et al.*, 1993), and plant pathogens (Bradley *et al.*, 1992). Therefore, it was studied if storage duration of lily bulbs at -2 □C is limited by oxidative stress. To find the primary injury of the tissue, assays on oxidative stress were carried out with visually undamaged scales or parts of scales directly after storage at -2 □C (Chapter 8).

Ion leakage from scales gave no indication that the plasma membrane integrity of the cells was affected until 4 years of storage. Contents of GSH and GSSG, and the glutathione

oxidation state did not change significantly over the first 3.3 years of storage. Moreover, during 5 years of storage, neither a decrease in the contents of phospholipids and neutral lipids, an increase in content of free fatty acids, nor an increase of the saturation level of phospholipid fatty acids was detected. On the contrary, the saturation level of phospholipid fatty acids decreased during storage, probably as a result of the low temperature. Although the results did not provide conclusive evidence for the absence of oxidative stress, it was concluded that no indication was found for oxidative stress in visually undamaged scales or parts of scales of lily 'Enchantment' bulbs until after a storage duration of about 4 years.

The lack of indications for the occurrence of oxidative stress during storage at -2 □C is a positive result from a horticultural point of view: because oxidative stress is thought to be a major process in senescence and ageing of plant tissues, its absence in lily bulbs during 4 years of storage gives good perspectives for prolonged storage.

Preservation of lily germplasm

Tested preservation methods

Generally, lily bulbs are stored in moist peat at -2 □C to extend the period of flower production (Beattie and White, 1993). Bulbs of ten genotypes could be propagated after 2 years of storage this way (Chapter 4), but those bulbs were no longer suitable for flower production. Bulb storage in moist peat at -2 □C was probably not limited by oxidative stress (Chapter 8). In our experiments, this storage was possibly limited by damage associated with *Penicillium* and/or extracellular ice (Chapter 8). Storage of partly dehydrated germplasm in polyethylene bags after a sufficient fungicide treatment could overcome both limitations and increase maximum storage duration. Storage under low O_2 and high CO_2 concentrations (controlled atmospheric storage = CAS) increased bulb quality (Peppelenbos and Van 't Leven, 1993) and could also increase maximum storage duration.

Scale bulblets of all ten genotypes survived 2 years of storage at -2 □C in air-tight sealed polyethylene bags (Chapter 5). Gas-permeable bags have also been successfully applicated for *in vitro* storage of strawberry germplasm (Reed, 1991). Advantages of storage in these bags compared to storage in moist peat are: a germplasm collection can be stored in relatively limited space, and scale bulblets can be stored dry, even partly dehydrated. Dry storage limits micro-organism growth, minimizes sprouting, and increases freezing tolerance

(Lång *et al.*, 1994; Maier *et al.*, 1994; Pearson and Davison, 1994). Also the reduced O_2 pressure in the polyethylene bags might increase freezing tolerance (Halloy and González, 1993). Unfortunately, the experiments on storage of bulblets in polyethylene bags were terminated before maximum storage duration was reached. The following approaches might give good prospects to determine the conditions for a storage duration of more than 2 years.

1) The optimal O_2 and CO_2 concentrations should be determined for lily scale bulblets stored at -2 □C. For lily bulbs stored at 10 □C, and used afterwards to produce flowers, the optimal O_2 concentration was between 2 and 5 % and the optimal CO_2 concentration was between 2 and 6 % (Peppelenbos and Van 't Leven, 1993). However, the optimal O_2 and CO_2 concentrations for scale bulblets stored at -2 □C might be different.
2) Research should concentrate on finding a MA package in which optimal O_2 and CO_2 concentrations will be established by scale bulblets at -2 □C.
3) Freezing tolerance of sealed lily scale bulblets should be determined and increased. When the storage temperature can be decreased, maximum storage duration will probably increase.

Sealed storage is probably also suitable for lily bulbs of larger size, but these bulbs are harder to protect against infection by fungi such as *Penicillium* than scale bulblets, because scale bulblets can easily be protected against pathogens by disinfection of scales before formation of these bulblets.

All ten genotypes survived 28 months of *in vitro* storage at 25 □C on medium with a quarter of the standard MS nutrients and 9 % sucrose (Chapter 6). The results indicated that longer storage than 28 months is possible. The medium with a quarter of the standard MS nutrients and 9 % sucrose also gave the best results at -2 □C, but bulblets of *L. longiflorum* and *L. henryi* died during *in vitro* storage at this temperature. A slightly higher temperature might be favourable for storage of lily germplasm *in vitro*, but might also induce leaf formation by breaking dormancy (De Klerk and Paffen 1995; Higgins and Stimart 1990). An *in vitro* collection has the advantage that the stored germplasm can be kept virus-free (Asjes, 1990). *In vitro* lily material is often used for research purposes, for instance for the development of methods for plantlet regeneration from protoplasts (Famelaer *et al.*, 1996; Mii *et al.*, 1994), the development of transformation methods (Langeveld *et al.*, 1995), and the

development of techniques for cryopreservation of meristems (Bouman and De Klerk, 1990; Matsumoto *et al.*, 1995).

The storage methods were tested using 8-10 lily genotypes. Because the genetic backgrounds of the genotypes used represents a wide genetic variation, it is expected that the storage methods tested are also suitable for other genotypes of lily.

Alternative preservation methods

Cryopreservation allows storage for almost unlimited periods (Withers, 1991). Apical meristems from scale bulblets of *L. japonicum* Thunb. survived cryopreservation for approximately 80 percent (Matsumoto *et al.*, 1995). However, the preparation of meristems is time consuming, and during freezing and thawing the meristems are easily damaged. Generally, cryopreservation has been most successfully used for cell cultures (Engelmann, 1991; Withers, 1991). Techniques for plant regeneration from cells and protoplasts have recently been developed for lily (Famelaer *et al.*, 1996; Mii *et al.*, 1994).

Another germplasm preservation method is storage of pollen (Hoekstra, 1995). Although pollen is generative material, there is no difference with storing vegetative material as to the results of crossings in a breeding program. Pollen storage has several advantages above storage of vegetative material.

1) Breeders can directly use pollen to make crosses in a breeding program, without having to regenerate the germplasm into flowering plants.
2) Pollen are smaller than bulbs, scales, and scale bulblets, which means that large numbers can be easily preserved (Hoekstra, 1995).
3) Pollen of lily can be stored for more than 10 years (Iwanami, 1984; Loewus and Loewus, 1990).

However, pollen preservation has also some disadvantages.

1) Pollen can only be used as male parent, which means that cytoplasmic inheritance will be missed (Hoekstra, 1995).
2) Pollen can not be screened for unknown features.
3) Harvest conditions are very critical, because pollen is very sensitive to water (Hoekstra, 1995).
4) A pollen sample can not be propagated to new pollen. Therefore, it remains necessary to preserve a vegetative lily germplasm collection in addition to pollen.

The International Plant Genetic Resources Institute (IPGRI) asks genebanks world wide to accept the responsibility for the long-term conservation of the base collections of given crops and to keep material in active collections under the International Undertaking on Plant Genetic Resources. This non-binding agreement has the aim to ensure that resources are identified, collected, conserved, evaluated and made available without restriction (Guarino *et*

al., 1995). Although lily is of increasing economical importance, there still is not a genebank, which bears the official responsibility for a lily germplasm collection coordinated by the IPGRI. Lily bulbs and scale bulblets are the most suitable material to maintain an active germplasm collection, and an *in vitro* germplasm collection can be used as both an active and a base collection. The storage methods developed in the present study, make the maintenance of a lily germplasm collection cheaper and more efficient. Therefore, they facilitate the start of a lily collection under the International Undertaking on Plant Genetic Resources.

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Summary

Germplasm collections are important for crop improvement and research. Lily genotypes must be preserved vegetatively as clones, because the genotypes are unique and heterozygous. Using seeds would affect the unique genetic combinations. Collections of bulb crops are usually maintained by yearly planting, harvesting, and storing of the bulbs. Eliminating one or more seasons of bulb growing by long term bulb storage would reduce costs for maintaining a lily collection. Therefore, research was started to develop techniques for long term storage of lily bulbs. The objectives of the experiments described in this thesis were:

1) The development of methods to measure viability.
2) The development of techniques for long term storage.
3) The development of techniques to increase freezing tolerance.
4) The determination of the involvement of oxidative stress in the loss of regeneration capacity during storage of lily bulbs in moist peat at -2 □C.

The viability of lily scales, onion bulbs and tulip bulbs was decreased artificially by frost, heath or drying out treatments. After these treatments, the material was placed in distilled water for 1.5 hour. Ion leakage was determined by conductivity and potassium leakage of external solution. Afterwards, the material was planted to observe growth and development. In all instances, severe damage or death of the material was accompanied by high values of conductivity and potassium leakage. Ion leakage measured by conductivity and potassium content of external solution after 1.5 h leakage of scales gave similar results (Chapter 2).

The viability of lily bulb scales of 'Avignon', 'Esther', 'Star Gazer', 'Snow Queen' was determined after storage at -2 □C for 0.5, 1.5 and 2.5 years. Ion leakage, the percentage of scales that formed bulblets and the number and weight of these bulblets were determined on scales from the inner, middle and outer part of bulbs. During storage, the outer scales of all cultivars and all scales of 'Snow Queen' showed increased ion leakage accompanied by a

decreased ability to form scale bulblets during storage. Concomitantly, the percentage of scales forming bulblets declined and more and smaller scale bulblets were formed per regenerative scale. It was concluded that, ion leakage is a useful criterion to measure viability of lily scales (Chapter 3).

The maximum storage duration of *Lilium* bulbs stored at -2 □C in moist peat was determined for 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Asiatic hybrids), 'Star Gazer' (Oriental hybrid), 'Gelria', and 'Snow Queen' (*L. longiflorum*). The viability was determined by the percentage of bulbs with at least one regenerative scale (bulb regeneration), the proportion of regenerative scales (scale regeneration), and ion leakage of white inner scales. Maximum storage duration based on bulb and scale regeneration varied between 2.9 and 4.0 years for the Asiatic hybrids and between 2.0 and 2.4 years for the other cultivars. Ion leakage of inner scales was increased for all cultivars at a storage duration of 3 years except for 'Enchantment' and 'Mont Blanc'. It was concluded that a lily collection can probably be effectively stored for 2 years at -2 □C in moist peat (Chapter 4).

Scale bulblets of 10 lily genotypes, including Asiatic hybrids, Oriental hybrids, *Lilium longiflorum*, and *L. henryi*, were stored either dry, sealed air-tight in polyethylene bags, or in moist vermiculite in open polyethylene bags for a period of 2 years at -2 □C, 0 □C and 17 □C. Storing scale bulblets air-tight in polyethylene bags at -2 □C resulted in the smallest decrease in mass, the least ion leakage and the highest sprouting proportion after 2 years of storage. All genotypes survived 2 years of storage this way (Chapter 5).

In vitro regenerated bulblets of 10 lily genotypes (Asiatic hybrids, Oriental hybrids, *L. longiflorum* and *L. henryi*) were stored for 28 months at -2 □C and 25 □C on four different media: a quarter or standard concentration MS-nutrients with 9 % (w/v) or 6 % sucrose. The combination of a quarter of the MS-nutrients and 9 % sucrose gave the highest reduction in sprout and bulb growth, the highest viability and the highest percentage of regrowth after 28 months of storage. At 25 □C, all lily genotypes survived 28 months of storage under these conditions. At -2 □C, Asiatic and Oriental hybrids survived 28 months of storage, whereas genotypes of *L. longiflorum* and *L. henryi* survived 6 months of storage, but died during prolonged storage at this temperature (Chapter 6).

Effects of freezing duration, previous storage duration of bulbs at -2 □C, and partial dehydration of scales on freezing tolerance of lily scales were studied for a series of cultivars. Freezing tolerance of scales was estimated by measuring ion leakage and recording scale

bulblet regeneration. Both methods gave similar results. Freezing tolerance decreased with freezing exposure. A longer previous storage duration of the bulbs at -2 □C tended to reduce freezing tolerance of the scales. Dehydration of the scales to 10-20 % loss of water content significantly increased freezing tolerance. Further dehydration to 30-40 % loss of water content did not further increase freezing tolerance. Nucleation temperatures, temperatures during crystallisation and melting temperatures of the scales were recorded for the cultivar 'Enchantment'. Nucleation occurred at higher temperatures after a longer previous storage duration of bulbs, indicating a reduced capacity to remain supercooled. The increased freezing tolerance of dehydrated lily scales could partly be explained by a decreased melting temperature of the scales. Long term storage of lily bulbs at -2 □C was concluded to be safer after partial dehydration to 10-20% loss of the original water content (Chapter 7).

Possible involvement of oxidative stress in the loss of regeneration capacity was tested for 'Enchantment' scales from bulbs stored for 0 to 5 years at -2 □C in moist peat. Regeneration ability decreased after more than 1 year of storage and was completely lost after 5 years. White (i.e. with no visual damage) scales were used to test whether breakdown of membranes by oxidative stress was an early event in this storage-induced viability loss of lily bulbs. Estimates of changes in ion leakage, the content and oxidation state of glutathione, the content of phospholipids, the content of neutral lipids, the content of free fatty acids, and the degree of unsaturation of fatty acids in phospholipids during storage, gave no indication that oxidative stress is a major factor associated with the loss of regeneration capacity of lily bulbs during cold storage (Chapter 8).

The developed storage methods, facilitate the maintenance of a lily germplasm collection. The possibility to increase freezing tolerance by partial dehydration, and the probable absence of oxidative stress during cold storage give good prospects for the development of techniques providing a further increase in the maximum storage duration of lily germplasm.

Samenvatting

Collecties van uitgangsmateriaal zijn belangrijk voor het verbeteren van gewassen en voor onderzoeksdoeleinden. Leliegenotypen moeten vegetatief worden vermeerderd, omdat ze uniek zijn en heterozygoot. Vermeerdering via zaad zou uitsplitsing van de genotypen tot gevolg hebben. Collecties van bolgewassen worden gewoonlijk in stand gehouden door jaarlijks planten, rooien en bewaren van bollen. Het bewaren van de bollen gedurende één of meer seizoenen zou deze in-standhouding een stuk goedkoper maken. Met dit doel werd onderzoek gestart naar lange-termijn bewaring van lelie. De onderwerpen van de verschillende experimenten die in dit proefschrift beschreven zijn, waren:

1) De ontwikkeling van methoden om vitaliteit te meten.
2) De ontwikkeling van technieken voor lange-termijn bewaring.
3) Het ontwikkelen van methoden om vorsttolerantie te vergroten.
4) Onderzoek naar de rol van oxidatieve stress bij het verlies aan regeneratievermogen gedurende het bewaren van leliebollen in vochtige turf bij -2 □C.

De vitaliteit van lelieschubben, en bollen van tulp en ui werd kunstmatig verminderd door vorst, hitte of uitdroging. Hierna werd het materiaal 1.5 uur in gedestilleerd water gelegd. Het plantmateriaal werd geplant om groei en ontwikkeling te meten en ionlekkage werd bepaald via de elektrische geleidbaarheid en het kaliumgehalte van het water. In alle gevallen ging dood en zeer slechte groei en ontwikkeling van het materiaal samen met hoge waarden voor ionlekkage. Ionlekkage gemeten via geleidbaarheid en via het kaliumgehalte van het water leidde tot overeenkomstige resultaten (Hoofdstuk 2).

Ionlekkage en het percentage schubben dat schubbolletjes vormde, en het aantal en gewicht van deze bolletjes werd bepaald voor bollen van 'Avignon', 'Esther', 'Star Gazer' en 'Snow Queen' na 0.5, 1.5 en 2.5 jaar bewaren bij -2 □C. Schubben werden genomen uit het binnenste, het middelste en het buitenste gedeelte van de bollen. Voor alle schubben uit het buitenste gedeelte en voor alle schubben van 'Snow Queen' nam ionlekkage toe met de bewaartijd en nam het regeneratievermogen van de schubben af: minder schubben vormden bolletjes en bij de schubben die nog wel bolletjes vormden waren deze talrijker, maar kleiner.

Er werd geconcludeerd dat het meten van ionlekkage nuttig was voor de bepaling van de vitaliteit van lelieschubben (Hoofdstuk 3).

De maximale bewaarduur van leliebollen in vochtige turf bij -2 □C werd bepaald voor 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Aziatische hybriden), 'Star Gazer' (Oriental hybride), 'Gelria', and 'Snow Queen' (*L. longiflorum*). Vitaliteit werd bepaald via het percentage bollen met tenminste één regenererende schub (bolregeneratie), het percentage regenererende schubben (schubregeneratie) en ionlekkage van binnenste schubben zonder zichtbare schade. Gebaseerd op bol-en schubregeneratie lag de maximale bewaarduur tussen 2.9 en 4.0 jaar voor de Aziatische hybriden en tussen 2.0 en 2.4 jaar voor de andere rassen. Ionlekkage was na 3 jaar verhoogd voor alle rassen, behalve 'Enchantment' en 'Mont Blanc'. De conclusie was dat een leliecollectie waarschijnlijk 2 jaar kan worden bewaard in vochtige turf bij -2 □C (Hoofdstuk 4).

Schubbolletjes van 10 leliegenotypen (Aziatische hybriden, Oriental hybriden, *L. longiflorum*, en *L. henryi*), werden gedurende 2 jaar bewaard bij -2 □C, 0 □C of 17 □C. De schubbolletjes waren droog verpakt luchtdicht in polyethyleen zakjes, of in vochtige vermiculiet in open zakjes. Luchtdicht verpakte bewaring bij -2 □C gaf de laagste massavermindering, de minste ionlekkage, en het hoogste uitgroeipercentage na 2 jaar bewaren. Alle genotypen overleefden 2 jaar bewaring via deze methode (Hoofdstuk 5).

Bolletjes van 10 leliegenotypen (Aziatische hybriden, Oriental hybriden, *L. longiflorum*, en *L. henryi*), werden gedurende 28 maanden *in vitro* bewaard bij -2 □C en 25 □C op vier verschillende voedingsmedia: een kwart of standaard concentratie MS-nutriënten met 9 % (w/v) of 6 % sucrose. Het medium met een kwart van de MS-nutriënten en 9 % sucrose gaf gedurende bewaring de laagste spruit- en bolgroei en de hoogste vitaliteit. Ook hergroei na planten in de kas na bewaring was het best bij dit medium. Alle genotypen overleefden 28 maanden bewaren bij 25 □C op dit medium. Alleen de Aziatische en Oriental hybriden overleefden 28 maanden bewaren bij -2 □C op dit medium, terwijl de genotypen van *L. longiflorum* en *L. henryi* slechts gedurende 6 maanden vitaal bleven bij deze temperatuur (Hoofdstuk 6).

Effecten van vriesduur, voorafgaande bewaarduur van bollen bij -2 □C, en gedeeltelijke uitdroging van schubben op vorsttolerantie van lelieschubben werden onderzocht voor verschillende rassen. Vorsttolerantie werd bepaald met behulp van ionlekkage en regeneratievermogen van schubben. Beide methoden gaven overeenkomstige

resultaten. Vorsttolerantie nam af met vriesduur en leek af te nemen met een langere voorafgaande bewaarduur van bollen bij -2 □C. vorsttolerantie van 10 tot 20 % gedroogde schubben was significant verhoogd. Uitdroging tot 30 tot 40 % watergehalteverlies verhoogde de vorsttolerantie niet verder. Nucleatie-, kristallisatie- en smelttemperaturen werden bepaald voor schubben van 'Enchantment'. De nucleatietemperatuur nam toe met een langere voorafgaande bewaarduur van bollen, duidend op een verminderde capaciteit om onderkoeld te blijven. De toegenomen vorsttolerantie van 10 tot 20 % ingedroogde schubben kon slechts ten dele worden verklaard door een verlaagd smeltpunt van de schubben. Er werd geconcludeerd dat 10 tot 20 % indrogen van leliebollen de kans op schade verkleint tijdens langdurige bewaring bij -2 □C (Hoofdstuk 7).

De mogelijke rol van oxidatieve stress bij het verlies aan regeneratievermogen werd onderzocht voor 'Enchantment' schubben van bollen die 0 tot 5 jaar waren bewaard in vochtige turf bij -2 □C. Het regeneratievermogen nam af na 1 jaar bewaren en was verdwenen na 5 jaar bewaren. Schubben zonder zichtbare schade werden gebruikt om te testen of oxidatieve afbraak van membranen een vroeg optredend proces was bij het genoemde verlies aan regeneratievermogen. Metingen aan ionlekkage, het gehalte en de oxidatie van glutathion, het gehalte en de samenstelling van fosfolipiden, neutrale lipiden en vrije vetzuren gaven geen indicatie dat oxidatieve stress een belangrijke factor is die samengaat met het verlies van regeneratievermogen gedurende bewaring van leliebollen bij -2 □C (Hoofdstuk 8).

De ontwikkelde bewaarmethoden maken de in-standhouding van een leliecollectie efficiënter. De mogelijkheid vorsttolerantie te vergroten door gedeeltelijke uitdroging en de waarschijnlijk geringe rol van oxidatieve stress gedurende koude bewaring bieden goede perspectieven voor het ontwikkelen van methoden, met een langere maximale bewaarduur dan de tot nu toe ontwikkelde methoden.

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Curriculum Vitae

Franciscus Johannes Martinus (Frans) Bonnier werd op 18 februari 1963 geboren te Rotterdam. In 1981 behaalde hij het VWO-B diploma aan het Titus Brandsma College in Dordrecht. In het zelfde jaar begon hij aan zijn studie Plantenveredeling aan de toenmalige Landbouw Hogeschool (nu Landbouwuniversiteit) te Wageningen. In 1985 behaalde hij het kandidaatsexamen met lof. De praktijktijd werd doorgebracht op twee locaties. Drie maanden werden doorgebracht op het veredelingsbedrijf 'Nickerson Zwaan' in Barendrecht en drie maanden werden doorgebracht op het toenmalige Instituut voor de Veredeling van Tuinbouwgewassen (nu opgegaan in DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek: CPRO-DLO). Het doctoraalexamen met de hoofdvakken Planten-veredeling en Erfelijkheidsleer en het bijvak Virologie werd in januari 1988 behaald. Van 1 juli 1988 tot 1 juni 1991 werkte hij aan verschillende projecten op de afdeling Groente en Fruit van CPRO-DLO. Van 1 juni 1991 tot 1 november 1994 heeft hij gewerkt aan het project "Lange-termijn bewaring van lelie, tulp en hyacint" in het kader van het door de overheid en bloembollenbedrijfsleven gefinancierde Urgentieprogramma Bollenziekte- en Veredelingsonderzoek, hetgeen leidde tot dit proefschrift.