Aspects of bulblet growth of lily *in vitro*

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Contents

Chapter 1

General Introduction

***Lilium* L.**

The genus *Lilium* is one of the *ca.* 220 genera belonging to the *Liliaceae* and comprises about 85 species including many ornamental species. Lilies are among the top 10 commercial flowers of the world. They have large flowers with attractive colors (Fig. 1) and an excellent vase life (Beattie and White). *Lilium* is characterized by an annual thermo-periodism and is widely distributed in the Northern Hemisphere. *Lilium* is classified into several divisions on the basis of geographical and genetic origin, and the position of the flowers. The species of this genus are taxonomically classified into seven sections (*Martagon, Pseudolirium, Lilium, Archelirion, Sinomartagon, Leucolirion and Oxypetalum*) (De Jong, 1974).

Fig. 1. *Lilium cvs* Santander (left) and Stargazer (right) that were used for the experiments described in this thesis.

Propagation and micropropagation of lily

Commercially grown cultivars are propagated by vegetative means to maintain genetic purity. The natural vegetative propagules are small bulblets, either produced above ground on the stems (bulbils) or underground on bulb scales (Kumar et al., 2006). For conventional commercial vegetative propagation, bulblets are produced from scales. Excised scales are kept in a plastic bag in moistened vermiculite and each scale produces 1-4 bulblets. This procedure is called ‘scaling’ (Fig. 2). Because the

speed is nonetheless relatively slow, introduction of newly bred cultivars still requires a long period of time (Langens-Gerrits and De Klerk, 1999).

Fig. 2. Propagation of lily via scaling; a) lily scale culture on peat, b) regenerated lily bulblets after 12 weeks.

Micropropagation has the potency to produce large numbers of high quality plantlets in a short period of time (George et al., 2008). Basically, micropropagation in lily is just like scaling but carried out *in vitro* on an artificial nutrient medium. The major advantages of micropropagation are that small scale-explants can be used, that scales excised from the new bulblets can be used as new starting material so that per year a few propagation cycles can be performed, and that infection by micro-organisms is avoided (Fig. 3).

Fig. 3. Lily micropropagation *in vitro;* a) lily bulb, scale and explants cultured *in vitro,* b) lily bulblets regenerated *in vitro* after 12 weeks (bulblets, leaves, roots and scale explant).

The drawbacks of micropropagation are the high costs per propagule and the small size of the produced propagules. The latter leads to suboptimal performance after planting in soil. In micropropagation of *Lilium*, many tissues can be used but bulb scales are the favorite explants (Van Aartrijk and Van der Linde, 1986; Bahr and Compton, 2004; Han et al., 2005). Scales of lily bulbs are swollen petioles. Lily scale fragments cultured *in vitro* regenerate bulblets consisting of scales that may or may not carry a leaf blade (Jásik and De Klerk, 2006).

Importance of bulb formation in tissue culture

Bulblets and other storage organs produced *in vitro* have properties that make them preferable propagules. They can be easily handled, transported and stored and they do not require an extensive acclimatization procedure after transfer to soil (Thakur et al., 2006). In food crops, tissue culture is being used for production of microtubers in potato (Vreugdenhil et al., 1994), bulblets in shallot (Le Guen-Le Saos et al., 2002), garlic (Ravnikar et al., 1993), and onion (Knypl, 1980; Keller, 1993), and tuberous roots in sweet potato (Wang et al., 2006). In ornamentals, bulblets are produced as a last step during micropropagation of tulip (Kuijpers and Langens-Gerrits, 1996), lily (Bahr and Compton, 2004), *Narcissus* (Staikidou et al., 2005), *Hyacinthus* (Takayama et al., 1991), *Muscari* (Saniewski and Puchalski, 1987), *Hippeastrum* (Ilczuk et al., 2005) and iris (Van der Linde and Schipper, 1992). In some bulbous crops, bulblets are generated 'automatically' under normal tissue culture conditions and no special measures have to be taken (lily, hyacinth), but in other bulbous crops (tulip, iris) only shoots are formed. In the latter case, a special treatment is used to achieve bulb formation from the shoots.

The size of the bulblets produced *in vitro* has a strong effect on performance after planting. Studies with direct field planting of bulblets produced *in vitro* have shown that small bulblets emerge slower, less uniform and to a smaller percentage (Lian et al., 2003). Furthermore, the growth after planting is determined by the initial bulb weight. The initial weight influences growth after planting in two ways. The weight of bulblets after a growing season is linearly related with the initial weight (Langens-Gerrits et al., 1996b). In addition, when bulblets are sufficiently large (> 300

mg), they increasingly sprout with a stem instead of a rosette (Langens-Gerrits et al., 2003a). When sprouting with a stem, growth of the bulblets in soil is much better (Fig. 4). The change from sprouting with a rosette to sprouting with a stem is related to a switch in ontogenetic development from juvenile to adult vegetative.

Fig. 4. Lily bulblet growth in soil (left: small bulblet growth without a stem, right: bigger bulblet growth with a stem).

Effect of plant growth regulators on the growth of storage organs

All classes of hormones were found to have some effect on the formation of tubers and bulbs (Vreugdenhil and Sergeeva, 1999). The major ones are discussed below.

Gibberellins

Gibberellins inhibit tuberization, cause stolons to elongate rather than to swell, and inhibit starch accumulation and the synthesis of tuber-specific proteins (Xu et al., 1998; Vreugdenhil and Sergeeva, 1999). The effect of the gibberellin-synthesis inhibitors on growth and development of oriental lily hybrids has been examined and with these growth retardants heavy bulblets were obtained (Kumar et al., 2005). The

effect of growth retardants (like paclobutrazol or ancymidol) was studied in *Lilium* plantlets growing in liquid culture. A significant increase in leaf chlorophyll, epicuticular wax, plant dry weight and bulb starch contents were found in plantlets treated with growth retardants. A similar increase in the number of leaves, roots and bulbs was also noted (Thakur et al., 2006). Three inhibitors of gibberellin biosynthesis (ancymidol, flurpirimidol, and paclobutrazol) promoted bulb formation and the percentage of bulbing in shallot (Le Guen-Le Saos et al., 2002). In *Hippeastrum* tissue culture, flurpirimidol not only influenced the propagation rate, but also the size of the newly developed bulblets. Explants cultured in media containing flurprimidol formed much bigger bulblets (Ilczuk et al., 2005).

Jasmonates (JA)

Exogenously applied JA induces or promotes tuber formation in potato, yam, and orchid, as well as bulb formation in garlic and narcissus. The putative role of JA in storage organ formation has been corroborated by reports on increased endogenous levels of JA in bulb and tuber forming plants (Jásik and De Klerk, 2006). In lily, JA promotes relative bulb weight (bulb weight as a percent of plantlet weight). Here it should be noted again that scales of lily bulblets regenerated *in vitro* may or may not carry a leaf blade. In lily JA strongly inhibited leaf blade formation without promoting absolute bulblet weight. JA significantly enhanced shoot and bulb development in garlic (Ravnikar et al., 1993). In *Narcissus triandus*, JA plays an important role in the formation and enlargement of bulbs (Santos and Salema, 2000).

Abscisic acid (ABA)

In lily, ABA promotes relative bulb weight (bulb weight as a percent of plantlet weight), but not the absolute weight: ABA strongly inhibits leaf blade formation without promoting absolute bulblet weight. When fluridone, an inhibitor of ABA synthesis is added, scale formation is inhibited completely, but is restored when ABA is added along with fluridone (Kim et al., 1994).

Effect of other factors on the growth of storage organs

Various other factors have been examined with relation to storage organ formation. First, these factors are dealt with. The next section deals with tissue culture factors that are usually not recognized but may be decisive for adequate bulblet growth.

Temperature

Both a moderate low temperature (15 ˚C) and a severe cold treatment (5 ˚C) have a profound effect. A moderate temperature (15 ˚C) is required for a phase change. During the development of lily, three ontogenic phases can be distinguished, *viz*., juvenile, vegetative adult and flowering phase (Langens-Gerrits et al., 2003a). In *in vitro* cultured lily bulblets, the transition from juvenile to vegetative adult is characterized by the development of a tunica-corpus structure with increased mitotic activity in the apical meristem, followed by stem elongation (Langens-Gerrits et al., 1996a). This step is related to the weight of the bulblets (Matsuo and Arisumi, 1978; Niimi, 1995; Langens-Gerrits et al., 2003a), and also to a moderate low temperature treatment (15 ˚C) (Ishimori et al., 2007).

In lily bulblets that are planted in the field, the bulblet growth per mg leaf DW (this is the sink activity of the bulblets) is sharply promoted by a preceding cold treatment of bulblets at 5 ˚C (De Klerk, 2009). It was concluded that a cold treatment is necessary to stimulate sink strength of the bulblets. In tissue culture of lily bulb formation occurs without a preceding cold treatment. Apparently other bulb-inducers (*e.g*., the high sucrose concentration in the medium) play a role. On the other hand, bulb growth *in vitro* is much less than bulb growth in soil (see below) and this may well be caused by the lack of cold treatment. In tulip, a cold treatment is also necessary to obtain bulblet growth (Rice et al., 1983).

Light

Light intensity and quality also influence the growth of bulblets. The fresh weight of bulblets was significantly greater in dark than in light in *L. longiflorum* (Leshem et al., 1982). In potato darkening of both roots and shoots strongly promoted tuber formation; the tubers were formed on the darkened part of the plant (Aksenova et

al., 1994). The influence of varying light treatments (blue, green, yellow, red, far-red and UV irradiation) on shoot and bulb induction was studied in tissue culture of hyacinths *in vitro*. Blue light stimulated growth and development of adventitious shoots and buds regardless of carbohydrate type, while the highest number of bulbs was obtained under red or white light (Bach and Świderski, 2000).When stem cuttings of potato plants were cultured under red or blue light, red-light-plants were thin, long, with very small leaves, and produced no or only few microtubers (after longer-lasting cultivation). Blue-light-plants remained short, thick, with large, well developed leaves and produced a significant amount of microtubers (Aksenova et al., 1994). With respect to day length, in *L. ancifolium*, 16 h d^{-1} darkness had the best effect with respect to bulblet formation and enlargement (Zhang et al., 2010).

Explant size

In lily, the size of the explant (= scale fragment) has a major effect on the size of the regenerated bulblet. The size of the explant has yet another effect: bulblets of the same size that have regenerated from a large or a small explant respectively may differ with respect to the ontogenic age. The bulblets that have regenerated from a large explant are often adult and the ones from a small explant usually juvenile, and they sprout with a stem or with a rosette respectively (Langens-Gerrits et al., 2003a). There are a number of ways how the size can influence the regenerating bulblet, including hormonal or nutritional influences.

Sucrose

Sucrose does not affect the number of regenerated bulblets, but the size of bulblets increases with increasing concentration of sucrose (Han et al., 2005). During *in vitro* culture, growth of the bulblets depends on the sucrose concentration (Yamagishi, 1998). It was reported earlier that large bulblets were obtained *in vitro* on medium with a high concentration of sucrose (Van Aartrijk and Van der Linde, 1986; Langens-Gerrits et al., 2003b). The increase in bulblet size with high concentration of sucrose (60-90g l^{-1}) was reported in many *Lilium* cultivars using different explants (Takayama and Misawa, 1982; Bonnier and Van Tuyl, 1997). The increase in bulblet

size was mainly due to an increase in starch and total carbohydrates. The ontogenic age of the regenerated bulblets is influenced by the sucrose-mineral ratio in the medium with a high ratio being promotive for the phase change. Especially phosphorus seems to be important but this has not been examined critically (Langens-Gerrits et al., 2003a).

Lilium bulbs accumulate storage polysaccharides. Starch is the major storage polysaccharide in *Lilium* bulbs. In *L. longiflorum*, 85% of the storage polysaccharides are starch and the remainder glucomannan (Matsuo and Mizuno, 1974). Starch is an important reserve carbohydrate found in many plant species in all types of storage organs: seeds, tubers, bulbs and corms. It is deposited as crystalline granules, which consist of two polysaccharides, amylose (20-30%) and amylopectin (70-80%). In plant storage organs, starch biosynthesis takes place within the amyloplast (Ji et al., 2003). With respect to the biochemistry, it is widely accepted that plastidic ADP-glucosepyrophosphorylase (AGPase) catalyzes the first step. AGPase utilizes glucose-1-phosphate (Glc-1-P) and ATP to form ADP-glucose (the substrate for starch synthase) and PPi, which serves as the direct precursor for starch synthesis. To date the AGPase enzyme has been extensively studied in many sink organs of plants (Jaleel et al., 2007; Kato et al., 2007; Mohapatra et al., 2009). In mungbean seeds, a steady sink activity of the enzymes controlling carbon flux entering the seed may be required to achieve a large seed size (Chopra et al., 2007).

Stress

Under stressful conditions, plants also tend to increase allocation to below-ground biomass and storage organs (Puijalon et al., 2008). In correspondence with this, a short period of abiotic stress (heat, cold, anaerobiosis) increases rhizome growth in *Alstroemeria* by ca. 100% (Pumisutapon et al., 2012).

Nutrition of the regenerating bulblet; how do medium components reach the bulblet?

The conditions in tissue culture seem to be optimal: water is abundantly available, high levels of organic and inorganic nutrients are added and the temperature

is favorable. Nevertheless, the growth of plantlets in tissue culture falls short of expectations and seems at best similar to growth in the field. This has been ascribed to poor long-distance translocation in the explants (De Klerk, 2010). Lily bulblets generated *in vitro* also show relatively poor growth. Growth in tissue culture is linear and in the field exponential, resulting in much heavier bulblets *ex vitro* (De Klerk et al 1992). It should be noted that the *ex vitro* conditions include an adequate, constant temperature and relatively poor lightning. There may be several reasons for the poor growth of bulblets in tissue culture:

- The sink activity is limited; in tissue culture of lily no cold treatment is applied in contrast to e.g. tulip. In the field, a cold treatment increases sink activity in lily (De Klerk, 2009).
- The bulblet requires hormonal factors for proper growth (ABA? JA? anti-gibberellins?) which are only available in complete plantlets growing in soil.
- The tissue culture microenvironment is stressful, e.g. because sucrose is toxic (Desjardins et al., 2009)
- The supply of nutrients is inadequate.

The latter factor will be elaborated below. First, the supply under natural conditions will be discussed.

Translocation under natural conditions

Inorganic nutrients

In plants growing in the field, inorganics are taken up from the soil by the roots. Movement of solutes from the soil into the cell walls of roots occurs by diffusion or by hitching with the mass water flow. The ions move in the apoplast and the symplast up to the xylem parenchyma cells. However, a suberized cell layer in the endodermis, known as the Casparian strip, effectively blocks the entry of water and mineral ions into the stele via the apoplast. To pass through this cell layer, the solutes have to move into the symplast. After passing the Casperian strip, they may again move both in the symplast and the apoplast. The Casperian strip prevents moving backwards. Next, the solutes are loaded into the xylem tracheary cells and are then taken with the water flow in the xylem to the shoot.

Carbohydrates

In plants growing in the field there are two main sources of carbohydrates: photosynthetically active leaves and degrading storage organs. Sucrose is the major transport mode for carbohydrates and long-distance transport from source to sink organs occurs in the phloem (Li et al., 2010). In the source, energy is necessary to move carbohydrates from producing cells into the sieve elements in the phloem. This movement is called phloem loading. In the sink, energy is essential for some aspects of movement from sieve elements to sink cells, which store or metabolize the sugar. This movement of photosynthate from sieve elements to sink cells is called phloem unloading (Taiz and Zeiger, 2002). Phloem functions differ according to organ location. At least three parts can be defined: collection phloem in source organs (minor veins), transport phloem (along the path from source to sinks) and release phloem in sink organs (Van Bel, 1993). Sink organs rely heavily on the delivery of carbohydrates through the phloem for growth and development. Besides sucrose, other sugars are found and sometimes may be as abundant as sucrose, depending on species. They include polyols and oligosaccharides of the raffinose family. Other nutrients, such as amino acids and organic acids, are also found (Zimmermann and Ziegler, 1975). The loading of sucrose at the source and unloading at the sink brings about differences in osmotic potential that lead to uptake of water in the sink and release in the source and thereby the water flow in the phloem.

Transport to regenerating bulblets cultured in vitro

The components that are being translocated into growing bulblets include carbohydrates and inorganic nutrients. Carbohydrates originate from the scale explant and from the medium (Langens-Gerrits et al., 2003b). The percentage bulb growth that can be attributed to uptake of medium-sucrose is constant over the full regeneration period: 45-50 % for large (3 x 15 mm) and 65-75 % for small (3 x 5 mm) explants.

It is usually taken for granted that medium components reach the target tissue in the explants (usually the growing areas) in adequate amounts. But how are they translocated? Generally, solutes (compounds dissolved in water) may be translocated in two ways: (1) by diffusion and (2) by hitch hiking with the water flow. Diffusion is

driven by random thermal agitation and is fast over short distance, but very slow over large distances. According to Flick's law of diffusion, diffusion over 1 meter takes 32 years, over 2 cm one week, and over 50 μm 2.5 sec. Therefore, plants use water flow in the vascular tissues for long distance transport (Taiz and Zeiger, 2002). It has been discussed in the previous section how compounds are translocated to growing areas in plants in the field. In micropopagation, the distances between the source, the medium, and the sink (the growing areas) are a few millimeters to a few centimeters. Diffusion over a distance of 4 mm takes about 9h (depending on the diffusion-coefficient) and 2 cm takes 8 days which is slow. In addition, a second factor that results in low translocation via diffusion is the small diameter of the tissue via which transport occurs. Therefore, to obtain adequate growth in tissue culture most of the long distance transport of nutrients (from the medium to the growing regions of the shoot or from the medium to the developing bulblets) should also occur via the vascular bundles (De Klerk, 2010).

Xylem

The movement of water in the phloem is driven by transpiration. The tissue culture conditions are very humid so transpiration is expected to be reduced. Transpiration of shoots cultured *in vitro* has only been measured few times and was found to be 50 $\mu l.cm^{-2}.d^{-1}$ (Tanaka et al., 1991) and 30 $\mu l.cm^{-2}.d^{-1}$ (De Klerk, 2010) This is a small percentage of the transpiration rate in the field. Calculations showed that this is just enough to support growth when the compounds enter the cut surface together with water and move together with the water upwards into the shoot. Experiments with dyes, though, showed that when the cut surface is healed after the wounding reaction, most of the dye stays in the tissue at the cut surface. Similarly inorganic and organic nutrients may be captured at the cut surface. Regenerating lily bulblets that have no leaves because of culture in the dark, will have even less transpiration and still show bulblet growth similar or even higher than light-grown bulblets. We therefore conclude provisionally that xylem transport does not play a major role in the growth of lily bulblets.

Phloem

About phloem functioning in tissue culture nothing is known. As noted above, water flow in the phloem -so also the movement of solutes- is brought about by the loading and unloading of sucrose. In lily tissue culture, phloem unloading occurs in the regenerating bulblets and the mechanisms are most probably the same as in bulblets growing under natural conditions. Loading may occur in scale explants or in the leaves where sufficient sucrose might have accumulated by photosynthesis and/or by translocation in the transpiration flow in the xylem. In lily scale explants, the scale itself also functions naturally as a source when being degraded. It is not known in which developmental stage scales in tissue culture are. Scales seem to be both sink and source: They definitely acts as sink during tissue culture since they increase in weight but they also act as source since a significant portion of the carbohydrates translocated to the regenerating bulblet is scale-carbohydrate (Langens-Gerrits et al., 2003b). In potato, ^{14}C-glucose is incorporated in starch when the potato is a sink and in sucrose when it is a source (Viola et al., 2007). It is questionable whether sufficient loading occurs in the regenerating bulblet itself, so the scale explant seems to play a major role. It should be remembered that -in agreement with this- the size of the scale explant determined the size of the regenerating bulblet (Langens-Gerrits et al., 2003b).

Scope of the thesis

Lily is propagated via *in vitro* methods and the common explant for lily micropropagation is the scale explant. In spite of availability of nutrients, water, light and proper temperature *in vitro*, the growth of lily bulblets regenerated *in vitro* is lower than the growth of lily bulblets *ex vitro*. There is little literature available on the elucidation of the growth of lily *in vitro* as a model crop for bulbous crops (De Klerk et al., 1992; Langens-Gerrits et al., 1996b; Langens-Gerrits and De Klerk, 1999; Langens-Gerrits et al., 2003b; De Klerk, 2009).

In **Chapter 2**, we set up a novel method to reduce the contamination percentage of lily scale explants. Lily is a geophyte and underground storage organs of lily are used for micropropagation, so there is a high number of microorganisms which increase contamination percentage during tissue culture of lily. A diluted NaClO

solution was added to the rinsing water and subsequently cross-contamination decreased significantly. In addition, excision of scales of lily bulbs submerged in diluted NaClO also decreased lily contamination by preventing the entering of contaminated fluid percentage. The results described in this chapter showed that the common sterilization procedure is not the proper way to sterilize explants *in vitro*.

In **Chapter 3**, a detailed study on the effect of scale explants on growth of lily bulblets *in vitro* was done. Scale explants as the main explants for tissue culture of lily plays a key role in the growth of lily bulblets *in vitro*. The size of scale explants influenced the growth of lily bulblets *in vitro*. Studies on apical and basal scale explants showed that the intensity of vascular bundles and the amount of starch granules influenced the growth of lily bulblet regenerated *in vitro*. In this chapter, we describe the development of vascular bundles in lily scale explants.

In **Chapter 4**, several moderate abiotic stresses were found to improve the growth of lily bulblets *in vitro*. Hot air, hot water, drought and anaerobiosis were studied with respect to their effect on the growth of lily bulblets *in vitro*. The aim of this chapter was to understand whether moderate stresses can enhance the growth of lily bulblets *in vitro*.

In **Chapter 5**, the effect of CO_2 removal from the headspace of tissue culture containers on photosynthesis of *in vitro* grown lily and *Arabidopsis* is examined. In absence of CO_2, the growth and F_v/F_m decreased in lily and *Arabidopsis* cultured explants. Staining with nitro blue tetrazolium, a stain for reactive oxygen species (ROS), showed that tissue cultured plants contained a low, but significant level of ROS. This level was strongly increased when CO_2 had been removed, probably due to the lack of electron acceptors in the photosynthetic electron transport chain. ROS were virtually absent in *ex vitro* grown plants. An overall discussion about all above mentioned chapters is presented in **Chapter 6**.

Chapter 2

Stringent treatments to reduce contamination increased growth of lily bulblets regenerating *in vitro*

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Abstract

In tissue culture of *Lilium*, the standard initiation procedure brings about substantial contamination in two ways. (1) When scales are detached from the mother bulb, microorganisms can enter via the wound. Contamination was strongly enhanced by the negative hydrostatic pressure within the scales by which nonsterile fluid was sucked up at detachment. The occurrence of a negative hydrostatic pressure was demonstrated by penetration (23.8 mm) of a solution of acid fuschine in the vascular tissues when scales were detached from a bulb submerged in the solution. Penetration was only 1.4 mm when the hydrostatic pressure had been removed by detaching the scale before submergence in the dye-solution. Contamination decreased strongly when the scales were detached from bulbs that were submerged in 0.03% NaClO. Evidence is presented here that this type of contamination was as expected endogenous, i.e., localized in the interior of the explant. (2) During the rinsing of scales after surface-sterilization, the rinsing water becomes contaminated with microorganisms that have not been killed during surface-sterilization. This caused cross-contamination. This type of additional contamination was controlled by rinsing in 0.03% NaClO instead of 'sterile' water. In our conditions, these initiation-related sources of contamination led to ca. 20% and ca. 25% contamination, respectively, of otherwise uninfected scales. Bulblet growth increased with 17% and 22% by reducing negative-hydrostatic-pressure related contamination and cross contamination, respectively.

Introduction

Several microorganisms have been identified as contaminants in plant tissue culture, in particular fungi, yeast and bacteria. Bacterial contamination is most common (Leifert et al., 1991; Leifert and Cassells, 2001). With respect to the topographical localization, contaminants may inhabit the surface of the tissue (epiphytic) or live within the tissue (endophytic). The former are for the greater part removed by adequate surface-sterilization but for the latter there is no easy treatment. The main obstacle in controling internal contaminants is that within the tissue, antibiotics etc. added via the medium do not reach a concentration sufficiently high to be effective. This is caused by general difficulties in uptake and transport of medium ingredients in tissue-cultured plants (De Klerk, 2010; De Klerk and Askari, 2012). In spite of this, many researchers and companies add antibiotics to the nutrient medium. When the antibiotics are omitted after a number of subcultures, the contaminants always "return". Addition of antibiotics is, however, helpful because they prevent overgrowing of the nutrient medium. Endophytic microorganisms may be beneficial to some extent (Hallmann et al., 1997), but usually they seem to be inhibitory (Long, 1988; Pirttilä et al., 2008).

At the time of collecting explants, contaminants are present at the surface of the tissue and within the tissue. Apart from improper handling by operators, for example, carelessness or inadequate flaming (Kunneman and Faaij-Groenen, 1987), there are during the initiation procedure two possible ways of infection that are as yet not or only little recognized.

(1) When the explant is excised from the stock plant, open vascular tissue is exposed to the nonsterile environment. Since the xylem has a negative hydrostatic pressure brought about by transpiration (Taiz and Zeiger, 2002), neighbouring fluids containing contaminants are sucked up after detachment (Van Meeteren, 1988). (2) Since it is not feasible to sterilize explants individually, they are processed in batches of 5 to 50 or more. Cross-contamination may occur, after the surface-sterilization with concentrated NaClO, during the rinsing of explants with sterile water. Usually, the explants are rinsed three times with sterile water (George, 1993; Pierik, 1997). Rinsing is done rigorously probably because researchers are afraid that NaClO affects plant tissues also at low concentration. This is, however, unlikely. Some researchers even

add low levels of NaClO during tissue culture to avoid flourishing of microorganisms (Teixeira et al., 2006; Yanagawa, 2007; Sawant and Tawar, 2011). Researchers ignore the possibility of cross-contamination during rinsing because there seems to be no feasible alternative procedure and because it is believed that the period during which cross-contamination may occur is too short to cause serious problems.

Organs growing underground like bulbs are notorious for contamination (Ziv and Lilien-Kipnis, 2000). The aims of the present study were to determine whether contaminants are introduced during the initiation step and if so, to reduce this contamination by using a low concentration of NaClO instead of water.

Materials and Methods

Standard tissue culture conditions

Field-grown bulbs (circumference 18-20 cm) of *Lilium* cv. Santander were harvested, cold-treated to break dormancy and stored at -1.0 °C until use. The procedure was according to (Aguettaz et al., 1990). Scales were surface-sterilized for 30 min in 1% (w/v) NaClO, rinsed for 1, 3 and 10 min with sterile water and after that stored until use in sterile water (on average for 1-2 h). Two explants of 7 x 7 mm were cut from the scales and placed with the abaxial side on 15 ml medium in plastic culture tubes (3.5 cm diameter). The medium was composed of macro- and microelements (Murashige and Skoog, 1962), 30 g l^{-1} sucrose, 0.4 mg l^{-1} thiamin, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar) and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). The explants were cultured at 25 °C and 30 $\mu mol.m^{-2}.sec^{-1}$ (Philips TL 33) for 16h per day. Contamination was scored at time intervals of 2-4 days for 6 weeks. After 11 weeks of culture, the regeneration percentage, bulblet number and fresh weight per bulblet were determined.

Minimal concentration of NaClO for decontamination of fluids

Twenty nonsurface-sterilized scales were kept for 3 h in 300 ml water to obtain contaminated water. To determine the minimal effective concentration of NaClO in fluids, increasing quantities of NaClO were added to this contaminated water to obtain increasing concentrations (0, 0.01, 0.03, 0.06, 0.1 and 1.5%, w/v) and the solutions

were stored for 24 h at room temperature. After that, 2 ml of fluid LB medium (Lysogeny Broth medium, a nutritionally rich medium used for growth of bacteria; Duchefa, Netherlands) was added to 2 ml from each NaClO concentration and incubated at 37 °C for 3 d. Then, bacterial growth was evaluated by visual inspection.

Estimation of cross-contamination

Sixty outer scales and 30 inner scales were sterilized for 30 min in one beaker with 1% NaClO solution plus a few drops Tween 20. Then the scales were divided into two groups (30 outer scales and 15 inner scales), distributed over two beakers (so per beaker 45 scales), rinsed three times (1, 3 and 10 min; the first group with sterile water and the second group with 0.03% NaClO), and then stored until use (1-2h) in water or 0.03% NaClO, respectively. The rinsing fluids were stored at 4 °C to examine contamination. Explants were prepared and cultured as indicated above. We monitored contamination of the scales during 6 weeks of culture. The percentage contamination due to cross-contamination was calculated with the following formula:

$$\text{Cross-cont.}\,\% = \frac{\text{ContWR\%} - \text{ContChlR\%}}{100 - \text{ContChlR\%}} * 100$$

in which "Cross-cont (%)" is cross-contamination % and "ContWR (%)" and "ContChlR (%)" the contamination percentages after rinsing with water and 0.03% NaClO, respectively.

Estimation of hydrostatic-pressure related contamination

Scales were detached from the mother bulb under streaming water or under streaming 0.03% NaClO and stored in water or 0.03% NaClO, respectively. They were surface-sterilized in the usual way (30 min in 1% NaClO), and rinsed three times with 0.03% NaClO. Explants were prepared and cultured as indicated above. Contamination was monitored for 6 weeks and the hydrostatic-pressure related contamination that occurs when scales are detached from the mother bulb was calculated according the following formula:

$$\text{ContHP}\ (\%) = \frac{\text{ContWD}\ (\%) - \text{ContChlD}(\%)}{100 - \text{ContChlD}\ (\%)} * 100$$

in which " ContHP (%)" is hydrostatic-pressure related contamination (%) and "ContWD (%) " and "ContChlD(%)" the contamination percentages after detaching scales from bulbs submerged in water and 0.03% NaClO, respectively.

Determination of contamination in the rinsing fluids

The rinsing fluids (water and NaClO solutions) were inoculated on LB solid medium in a 9-cm Petri dish and 30 ml LB liquid medium in plastic test tubes (Duchefa, Netherlands). On the solid medium, 25 µl was inoculated and on the liquid medium 30 ml. Bacterial growth was determined after 3 d in dark at 37 °C.

Determination of transpiration by scales

Scales were detached from the bulbs, transferred to plastic culture tubes (3.5 cm diameter) with 10 ml water solidified with 0.7% agar and kept standing upright with their basal part on the medium. A layer of 1.5 mm paraffin oil was carefully added to prevent evaporation from the solidified medium. The weight of container + medium + scale + paraffin oil was determined every 60 min and the weight loss was taken as transpiration by the scale. There was negligible weight loss when no scales were present (less than 0.1 µl per hour) showing that almost all weight loss occurred via the scale.

The surface of scales was measured as follows. The abaxial and adaxial side of scales were traced on paper taking into account the curvature of the scales. Then the drawn surfaces were cut out with scissors and weighed. The surface was calculated using the weight of 1 cm^2 paper.

Visualization of stomata in lily bulb scales

The stomata were visualized by preparing epidermal impressions of the adaxial and abaxial surfaces of lily bulb scales. The scale impressions were made with polyvinylsiloxane based high precision President Light Body impression material (Coltène/Whaledent AG, Altstätten, Switzerland). The lily scales were detached from lily bulbs and pushed onto the semi-soft impression material with the abaxial and adaxial surface. After 5 minutes the impression materials had solidified and scales were removed. Then colourless nail polish was painted on the imprints. Dried nail polish peels were stripped off, placed on a microscope slide and observed with an Axiophot light microscope (Zeiss, Oberkochen, Germany). Images were taken with an AxioCam ERc5S digital camera (Zeiss).

Determination of water movement into the vascular tubes caused by the negative hydrostatic pressure

To show that fluid can penetrate easily far into the scale explants by the negative hydrostatic pressure, the dye acid fuchsine was used as a visual marker. Scales were detached from bulbs that were submerged in 0.5 % acid fuchsine and kept submerged for 5 sec. As a control, scales were detached in the normal way and after 2 sec submerged in acid fuchsine for 15 sec. Each treatment was represented by 5 scales and each scale was dissected from the bottom upwards until no more color agent was detected by eye and binocular.

Statistics

In the figures, the means are shown ± SE. The statistical significances of differences in percentages and means were evaluated by the χ^2 and by the Student *t*-test, respectively. For each treatment, at least 50 explants were taken. Each experiment was carried out at least twice.

Results

Determination of the minimal concentration of NaClO for decontamination of fluids

Bacteria did grow in LB medium to which no or 0.01% NaClO had been added (Table 1). The lowest NaClO concentration that fully inhibited bacterial growth was 0.03%. Thus, a 0.03% solution of NaClO is suitable to prevent contamination after the surface-sterilization, provided the tissues are not being damaged.

Table 1. Minimum concentration of NaClO for disinfection of fluids.

		NaClO concentration (%)					
		0	0.01	0.03	0.06	0.1	1.5
LB liquid medium	1	+++	++	-	-	-	-
	2	+++	++	-	-	-	-
	3	+++	++	-	-	-	-

To contaminated fluid, a concentrated solution of NaClO was added. After 2 d at 25 ºC, liquid LB was and after another 3 d at 37 ºC, bacterial incidence was scored. (- not contaminated, ++ moderately contaminated, +++highly contaminated).

Estimation of cross-contamination caused by rinsing with water

NaClO remaining from surface-sterilization was removed by rinsing with water or with diluted NaClO (0.03%). The presence of microorganisms was examined both in the rinsing fluids (Table 2) and in the scale tissues (Fig. 1). The rinsing fluids were examined with solid and liquid LB. No contaminants occurred in NaClO rinsing solutions but they were present in rinsing water (Table 2). Bacteria were present in the 3rd rinsing water and in the storage water as shown with both solid and liquid LB and in the 2nd rinsing water as shown with liquid LB. The bacterial contaminants in the rinsing and storage water expectedly bring about cross- contamination. Cross-contamination can not occur in 0.03% NaClO since at this concentration all contaminants were killed. The 1st rinsing water contained no contamination probably because of a low concentration of NaClO due to carry-over from the surface-sterilization. Contamination after surface-sterilization can be attributed to incomplete surface-sterilization, endogenous contamination or cross-contamination during rinsing. We assumed that most cross-contamination occurs from outer scale explants (often endogenously contaminated) to inner scale explants (hardly endogenously contaminated). Evidently, there might also be cross-contamination from contaminated to noncontaminated outer scales. In inner scales, the percentage contamination decreased from 27% after rinsing with water to 3% after rinsing with NaClO (Fig. 1).

Table 2. Contamination of rinsing fluids as detected with LB solid (SM) and liquid (LM) medium.

		Water				**NaClO**			
		1st rinse (1 min)	2nd rinse (3 min)	3rd rinse (10 min)	Storage (120min)	1st rinse (1 min)	2nd rinse (3 min)	3rd rinse (10 min)	Storage (120min)
Test SM	1	-	-	+	++	-	-	-	-
	2	-	-	+	++	-	-	-	-
	3	-	-	+	++	-	-	-	-
Test LM	1	-	+	++	+++	-	-	-	-
	2	-	+	++	+++	-	-	-	-

In rinsing and storage fluids, contamination was examined with solid and liquid LB. The tests were done 3 and 2 times, respectively. Bacterial incidence was scored after 3 d at 37 ºC. (- not contaminated, ++ moderately contaminated, +++highly contaminated).

In this case, most of the contamination in water-rinsed scales was due to cross-contamination during the rinsing. About 25% of the previously uninfected inner scales were cross-contaminated. Rinsing outer scales with 0.03% NaClO reduced the

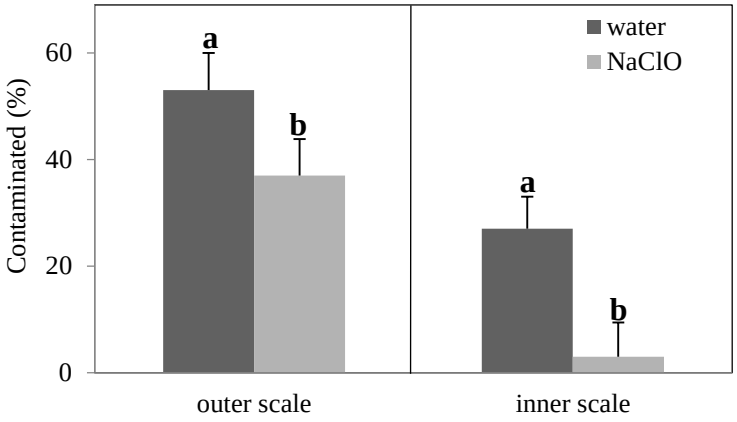

Fig. 1. Contamination of explants cut from inner and outer scales rinsed after the surface-sterilization with 1% NaClO with water or with 0.03% NaClO. Contamination was monitored for 6 weeks.

contamination from 53% to 37% (Fig. 1). A similar calculation as done for inner scales showed that in this case cross-contamination also occurred in about 25% of the otherwise noncontaminated outer scales. Inner scale explants showed lower contamination than outer ones: when rinsed with water 27% *vs.* 53% and when rinsed with 0.03% NaClO 3% *vs.* 37%. Contamination in outer scales is high because these scales are often somewhat damaged and because they are much older.

After 11 weeks of culture, FW (fresh weight) of lily bulblets regenerated from scale explants rinsed with sterile water or NaClO solution was measured. The growth was enhanced significantly by 22% in NaClO solution (Fig. 2a). In addition, there were no significant differences between the bulblet numbers (Fig. 2b) and the regeneration percentages (Fig. 2c) for the two rinsing solutions.

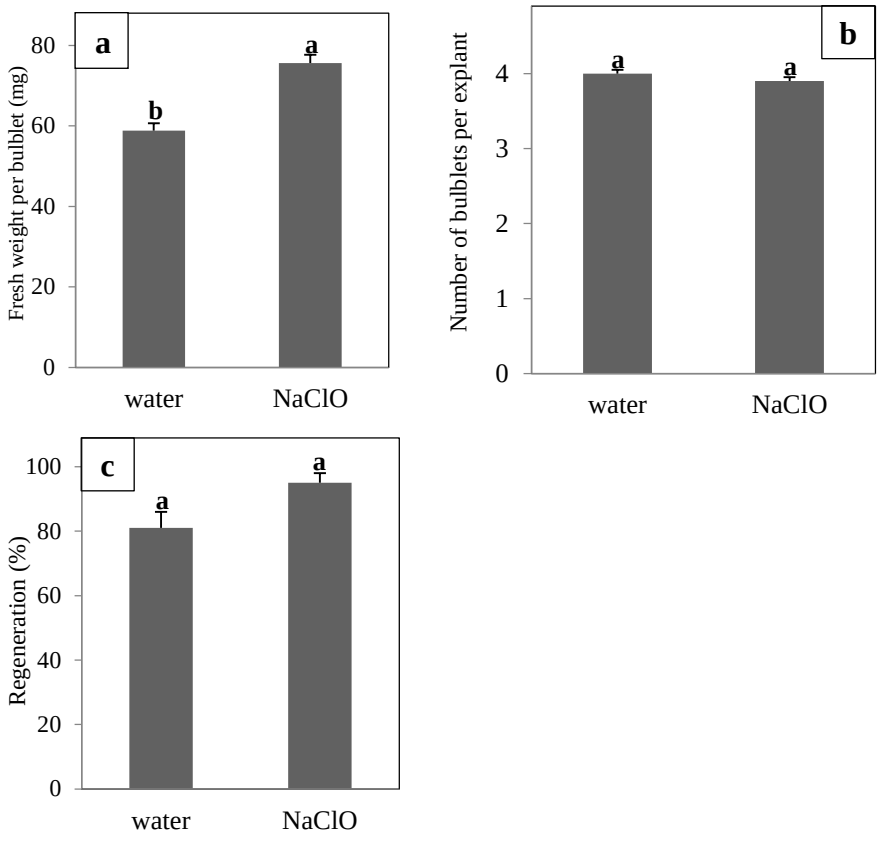

Fig. 2. Performance in vitro of explants cut from inner and outer scales rinsed after the surface-sterilization with sterile water or with 0.03% NaClO. The various parameters were determined after 11 weeks of tissue culture. Diluted NaClO (0.03%) had a significant effect on bulblet growth (a), but not at the number of regenerated bulblets (b) and not at the percentage of explants regenerating bulblets (c).

Estimation of hydrostatic-pressure related contamination

The xylem in shoots has a negative hydrostatic-pressure caused by transpiration. To the best of our knowledge, the rate of transpiration by subterranean organs like bulbs, if any, is not known. We determined for lily scales a transpiration rate of 10 µl per hour which equals 2.5 µl.cm^{-2}.h^{-1} (Fig. 3). In cacti, transpiration is 5-15 µl.cm^{-2}.h^{-1}

(Larcher, 1995). As expected, stomata were found (Fig. 4). They occurred on both the adaxial and the abaxial side of the scales.

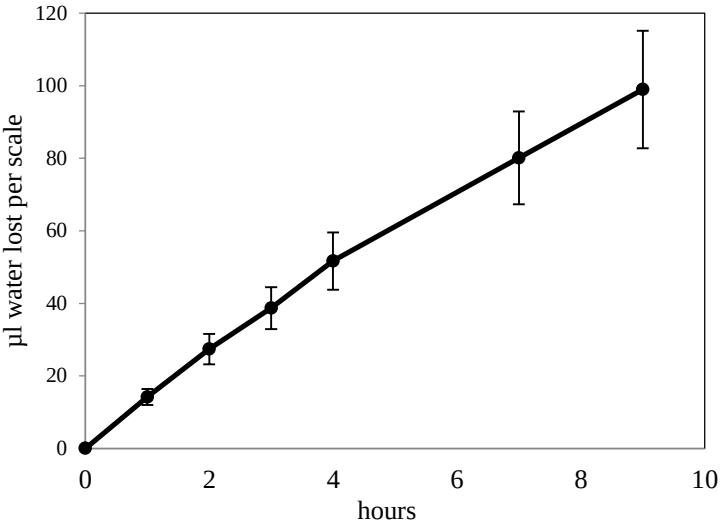

Fig. 3. Transpiration from lily scales at ambient humidity (48%) and temperature (21 ºC).

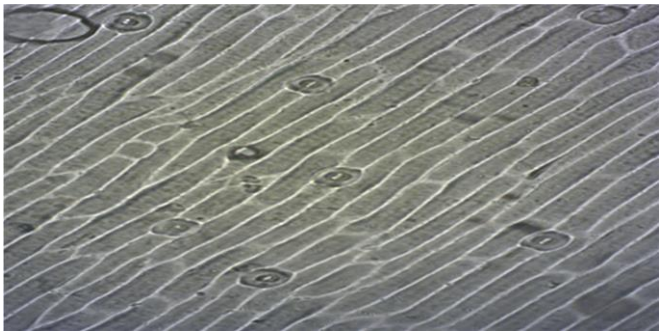

Fig. 4. Stomata located on the adaxial side of a lily bulb scale.

When scales were detached from bulbs submerged in an acid fuchsine solution, the dye penetrated into the bulb scale for 23.8 mm (Table 3, Fig. 5). In scales first

detached from the bulbs (so that the negative hydrostatic pressure was removed) and then kept for some seconds in the acid fuchsine solution, penetration was only 1.4 mm.

Table 3. Average distance (mm) of penetration of acid fuchsine in scales.

	Distance (mm)	**Penetration (%)**
Scales detached from bulbs submerged in dye-solution	23.8±2.8	47.4±4.6
Scales detached from bulbs prior to submergence in dye-solution	1.4±0.2	2.8±0.5

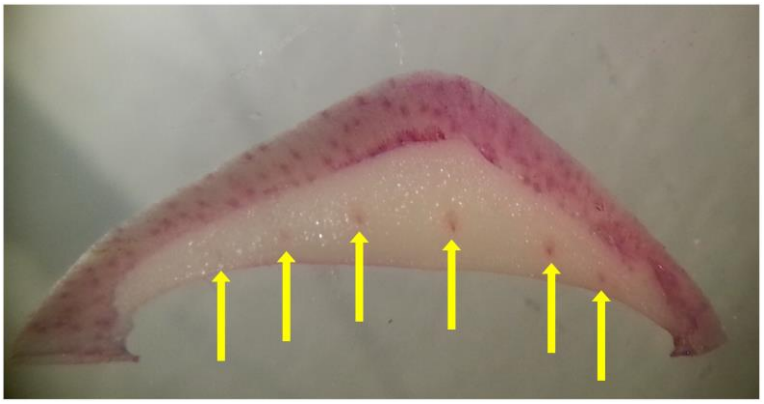

Fig. 5. Acid fuchsine penetrated into scales when the scales had been detached from bulbs submerged in an acid fuchsine solution. This shows the occurrence of a negative hydrostatic pressure in the vascular tissues.

Detaching the scales from the bulbs in diluted NaClO instead of water reduced contamination from 41% to 25% and from 55 to 37% in two consecutive experiments respectively (Fig. 6). Contamination by sucking up nonsterile water into the vascular tissues occurred in about 20% of the previously uninfected inner scales. In explants excised from water-collected scales, the period during which contaminants started to grow outside the explant lasted much longer than in NaClO-collected scales. Therefore, we scored visible contamination occurring in the first week and contamination that

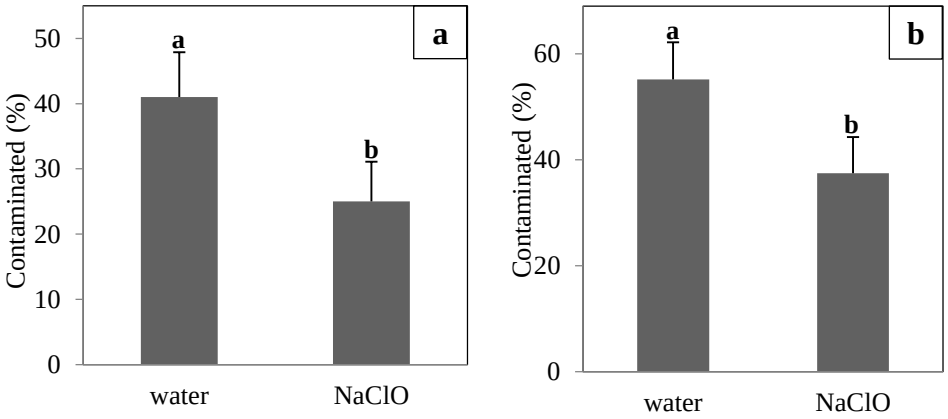

Fig. 6. Contamination of explants cut from inner and outer scales after detaching the scales in streaming water or streaming 0.03% NaClO. Contamination was monitored for 6 weeks. The results of two experiments are shown. a) 1st experiment; b) 2nd experiment.

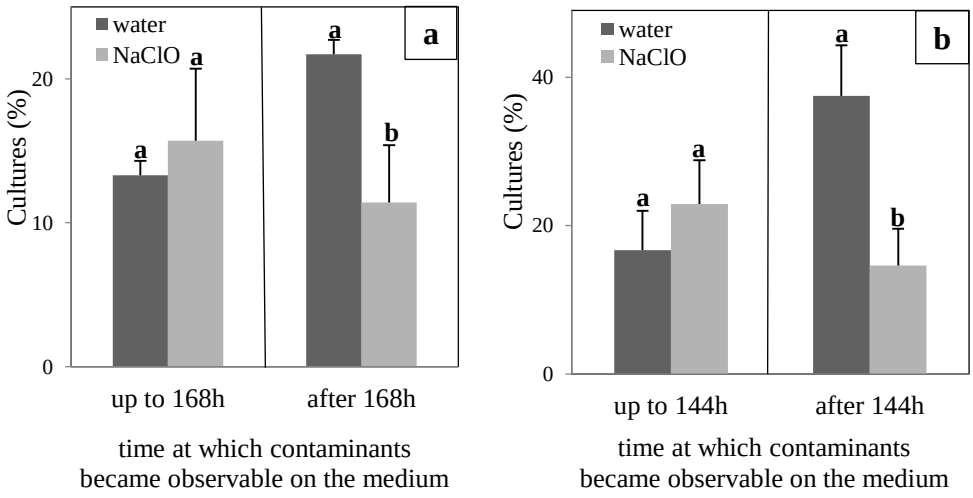

Fig. 7. First appearance of contamination with explants cut from inner and outer scales after detaching the scales in streaming water or streaming 0.03% NaClO. Contamination was monitored for 6 weeks and the appearance during the first week (up to 168 or 144h) or after that is shown. The results of two experiments are shown. a) 1st experiment; b) 2nd experiment.

appeared after the first week. During the first seven days, the contamination in NaClO-collected and water-collected scales was the same, but after that the water-collected scales showed higher contamination than the NaClO ones (Fig. 7).

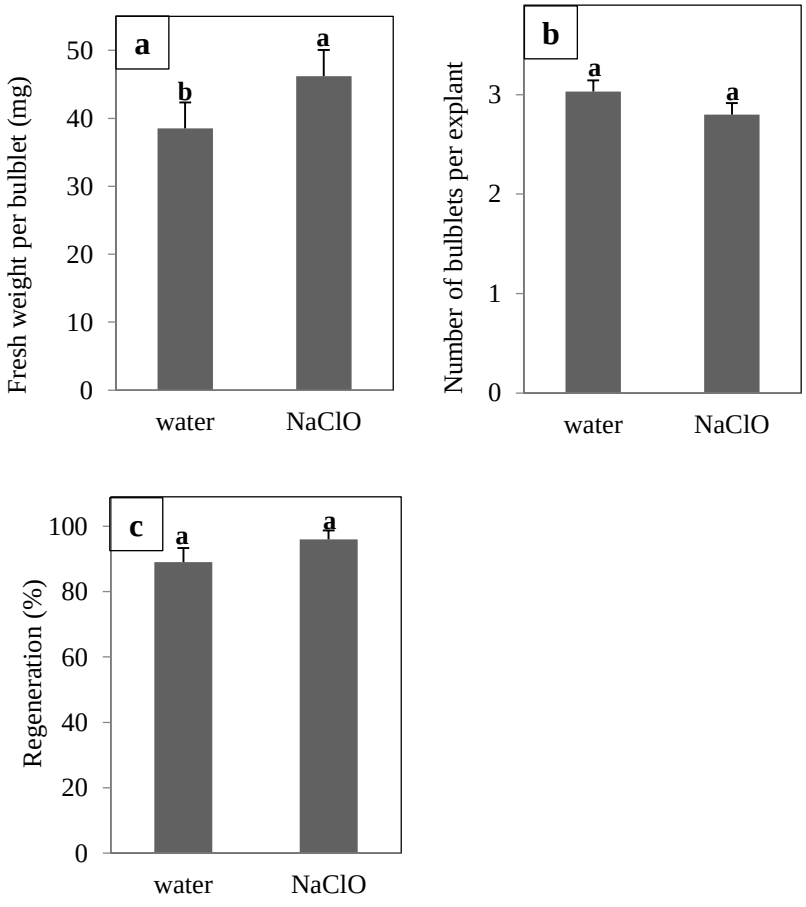

Fig. 8. Performance *in vitro* of scale-explants after detaching scales in streaming water or streaming 0.03% NaClO. The various parameters were determined after 11 weeks of tissue culture. a) Fresh weight per bulblets; b) Number of bulblets per explant c) and percentage of explants regenerating bulblets.

We assume that the period before contaminants started to grow outside the explant reflects the topographical localization of the contaminants. Thus, the contaminants that became visible after one week of culture were located more towards the interior of the explants.

Possibly, the early penetration of 0.03% NaClO into the tissue might inhibit regeneration or the growth of the regenerated bulblets. This was not the case (Fig.

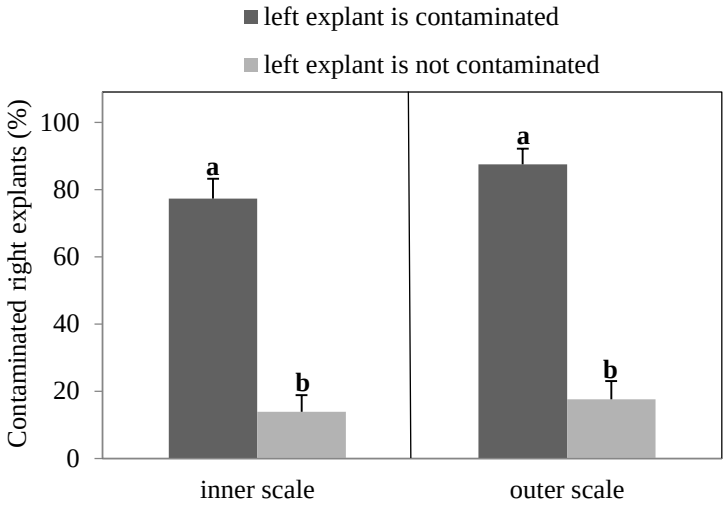

Fig. 9. Correlation of contamination between explants cut from one scale. From scales two explants were cut, one at the right side and one at the left side. Contamination was monitored for 6 weeks. The diagram shows the contamination percentage of the right explant when the left explant was or was not contaminated.

8a,b,c). As a matter of fact, bulblet growth enhanced significantly by the NaClO treatment with (17% , Fig. 8a).

Correlation between right and left explant contamination

We were interested whether explants excised from the same scale have a similar extent of contamination. To examine this, from each scale two explants were excised, one at the left side and the other at the right side. When in the inner scales the left explant was contaminated, 77% of the corresponding right explants were contaminated. When the left explant was not contaminated, from the corresponding right explants only 14% was contaminated (Fig. 9). The difference is highly significant. For the outer scales, these percentages were 88% and 15% respectively, also highly significant.

Discussion

Contamination is an everlasting problem in plant tissue culture. Apart from inadequate operating during manipulation in the laminar flow cabinet, poor equipment (*e.g.*, damaged filters in the laminar flow cabinet) and contamination by micro-arthropods (mites and thrips), the source of contamination is the explant that is transferred into tissue culture. In the present paper, we show in lily that explant-related contamination is caused to a substantial extent by faulty procedures during initiation. This may lead to both surface-localized and endogenous contaminants.

Explant-related contaminants may be surface-localized but difficult to kill by disinfectants probably because they are not adequately exposed to the disinfectant solution. Explant-related contaminants may also be internal and because of this not reachable by disinfectants. The latter is caused, among others, by the very slow long-distance translocation of solutes when driven only by diffusion (Taiz and Zeiger, 2002). In this case, only a heat treatment may be successful (Langens-Gerrits et al., 1998). Endophytic bacteria are reported to colonize intercellular spaces and vascular tissues and only a few reports demonstrate intracellular colonization (Hallmann et al., 1997).

In the experiments, we used a low concentration (0.03%) of NaClO for additional disinfection. This concentration was effective (Table 1) and is also reported to be adequate in medical practice (Heling et al., 2001). It should be noted that the target bacteria are moving freely in fluid and are therefore vulnerable. Accordingly, we could use a much lower concentration of NaClO than the one used for surface-sterilization.

First, we examined cross-contamination during the sterilization/rinsing procedure. When tissues from field-grown plants are surface-sterilized, a batch of a few to tens of explants is processed in one beaker because it is unfeasible to process the explants individually in a large number of beakers. When NaClO is present (during the surface-sterilization itself), cross-contamination is not possible. However, during the three rinses with sterile water that are commonly used to remove the excess of NaClO (George, 1993; Pierik, 1997) and the storage up to processing, cross-contamination may occur. When the scales were rinsed with sterile water for the 2^{nd} and 3^{rd} time, the rinsing water became heavily contaminated with bacteria (Table 2). This resulted in

considerable additional contamination of the explants. A simple way to reduce cross-contamination was rinsing with 0.03% NaClO instead of water. After rinsing in a diluted NaClO solution, lily bulblet growth enhanced significantly by 22% but regeneration % and number of bulblets regenerated *in vitro* had no significant difference between. The low toxicity (or the absence of toxicity) of a low concentration of NaClO corresponds to studies in which tissue culture was performed in the presence of a low concentration of NaClO (Teixeira et al., 2006; Yanagawa, 2007; Sawant and Tawar, 2011). Rinsing in diluted NaClO may also be considered for other crops.

The second target of the present study is the open connection between the vascular tissues and the environment when the scales are detached from the mother bulbs. This allows movement of microorganisms into the vascular tissue. Entering is strongly enhanced by the negative hydrostatic pressure in the tissue which results in sucking up of fluids close to the xylem just after excision. These fluids likely contain microorganisms. The xylem has a negative hydrostatic pressure because of transpiration of water from the leaves. In the case of lily bulbs, it should first be considered whether bulbs do have such negative hydrostatic pressure. Bulbs are underground and therefore may not display transpiration. On the other hand, bulbs are modified leaves (in the case of lily swollen petioles) so they are likely to have stomata. Microscopic inspection demonstrated the presence of stomata at both the abaxial and adaxial side (Fig. 4). Water was transpired at a similar rate as in cacti (Fig. 3). As a result, the xylem in lily scales will suck up liquid and contaminants may penetrate into the interior of the scale via this water flow. Provisional calculations showed that with a transpiration rate of 10 µl per scale per hour, water would penetrate in one hour more than 0.5 - 1 cm in addition to the intial penetration just after excision. The occurrence of a negative hydriostatic pressure was also shown by the deep penetetration of a dye when scales were detached from a bulb submerged in the dye solution.

As a result, contaminants penetrate so far into the interior of scales that disinfectants cannot reach them later on. It is important to note here that the diameter of xylem vessels is 10 to 100 µm and that the diameter of bacteria is a few µm. Thus there are no obstacles with respect to sizes. Flagellated bacteria may move actively reaching

a speed of over 1 m per hour (Schneider and Doetsch, 1974) and penetrate the tissue in this way.

We used again 0.03% NaClO to control these contaminants. Indeed, when the scales were during detachment submerged in 0.03% NaClO, contamination decreased in outer and inner scale explants by 15-20% (Fig. 5). Interestingly, in the scales that had been transferred to water and NaClO, the contamination that became visible during the first week was the same but after that, the water-collected scales showed significantly higher additional contamination. Obviously, the more contaminants are located in the interior of the explant, the longer it will take them to reach the nutrient medium. This unexpected finding was reproduced in a second experiment (Fig. 6).

We conclude that after detachment bacteria invade the xylem. When scales are collected in water or in air, these bacteria are transferred to tissue culture. After that, they will gradually exit the tissue and flourish on the nutrient medium. However, after some days or weeks, the wound is repaired and a layer of periderm has been formed. This inhibits uptake of compounds from the medium (Smulders et al., 1990) but also release of microorganisms from the tissue into the medium. So the microorganisms become trapped in the tissue and because of their detrimental effect, growth of the regenerating bulblets may be reduced (Fig. 7). On the other hand, when NaClO has penetrated the xylem at detachment, it will kill the bacteria. NaClO may itself have a negative effect on growth but is presumably short-lived within the tissue. Various compounds promote the decomposition of NaClO, among others various metal ions that are administered with MS. Most notably, chemical interactions between chelating agents and NaClO result in a rapid loss of free available chlorine (Rossi-Fedele et al., 2012) and both plant tissues and MS contain chelating compounds. Thus, within a few days/weeks, NaClO is probably fully decomposed.

To our knowledge, the problem of exposure of xylem tubes in the cut ends to penetration of contaminants has only been dealt with by (Thakur and Sood, 2006). They report that when sterilizing bamboo, tea and rose shoots with a length of 30-50 cm instead of a few cm, the contamination percentage drops from 50-60% to 20-25%. Apparently, in the long shoots the distance is too large for the penetrating microorganism to reach the upper part of the shoot that is transferred to tissue culture.

In both expriments, 0.03% NaClO did not inhibit regeneration. The growth of bulblets showed increases of 17% and 22% by reducing negative-hydrostatic-level related contamination and cross contamination, respectively.

In tissue culture of lily, substantial contamination may be caused during the initiation, both by the entrance of microorganisms directly after detachment of the scales (ca. 20% extra contamination in our conditions) and during the rinsing after surface-sterilization (ca. 25% extra contamination in our conditions). Both are effectively prevented by a diluted solution of NaClO (0.03%). These effective measures in lily are most probably also suitable for other species.

Chapter 3

The influence of scale explants on lily bulblet growth *in vitro*

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Abstract

Lily scales cultured *in vitro* regenerate bulblets that are used as propagules. We examined the influence of scale explants on the growth of regenerating bulblets. A large scale explant (6x18 mm) improved bulblet growth by 26% as compared to a small scale explant (6x6 mm). The beneficial effect of the scale became also clear when bulblets excised from scale explants were transferred to fresh medium for additional growth. When a small piece of the original scale was left attached to these bulblets, growth improved by 33%. The growth of lily bulblets was highest in explants cut from the middle of a scale (as opposed to the edge) and in explants cut from the basal half (as opposed to the apical half). In the basal scale explants, the number of starch granules was far higher than in apical scale explants. During culture *in vitro*, the number of vascular bundles increased in basal and apical scale explants from 6 and 3.3 to 8 and 4 bundles, respectively. The scale explants were physiologically very active during the culture *in vitro* as shown by a several fold increase in weight.

Introduction

Lily is one of the most important ornamental crops worldwide, both as a cut flower and as a flower bulb (Grassotti and Gimelli, 2010; Van Tuyl and Arens, 2010). Lily bulb scales are the common starting material in *in vitro* and *in vivo* lily propagation. After planting the new bulblets in soil, they should grow fast and produce plants with high quality. The performance of *in vitro* bulblets after planting depends among others on endogenous factors, including bulblet size, maturation and dormancy status (Langens-Gerrits et al., 2003a). The size of lily bulblets produced *in vitro* strongly influences performance after planting. Studies with direct field planting of *in vitro* bulblets have shown that small bulblets emerge slower and less uniform and have a lower sprouting percentage (Yae et al., 2001). In tulip, large bulblets regenerated *in vitro* also show better performance compared to small bulblets after transfer to soil (Le Nard et al., 1987; Hulscher et al., 1992). In addition, *in vitro* lily bulblets in the adult phase sprout with a stem and switch to a reproductive state; on the other hand, bulblets sprout with a rosette in the juvenile phase. Large bulblets are more often in the adult phase as compared to small bulblets (Langens-Gerrits et al., 2003a).

As compared to the growth of bulblets *in vivo*, the growth of lily bulblets *in vitro* is less than expected (De Klerk et al., 1992). A major, often overlooked feature of *in vitro* growth is that some *in vitro* conditions are far from optimal (e.g., the gaseous composition of the head space) and put plants under heavy stresses. It is generally disregarded that the size of bulblets *in vitro* is not only related to medium composition, but also to transportation of medium components to the newly regenerated bulblets. *In vitro*, vascular tissues have the major role in the transport of solutes from the medium to the areas of growth (De Klerk, 2016). Uptake of sucrose mainly occurs through the cut surfaces since the epidermis is relatively impermeable because of its wax layer. Moreover, according to (Langens-Gerrits et al., 2003b), sucrose taken up in the explants is mainly recovered at the basal side of the explants, where regeneration occurs. This indicates that internal transport of sucrose from the apical to the basal side may play a role in bulblet growth *in vitro*. The metabolism of sucrose is important to the growth and development of geophytes because sucrose metabolism enhances the growth of storage organs, inflorescences and other organs (Matsuo and Mizuno, 1974;

Wozniewski et al., 1991; Miller, 1992). In *L. longiflorum*, 85% of the storage polysaccharides are starch and the remainder consists of glucomannan (Matsuo and Mizuno, 1974). In plant storage organs, starch biosynthesis takes place within the amyloplast (Ji et al., 2003).

In this study we consider the influence of explants on the growth of lily bulblets during *in vitro* culture. We aim to reveal the mechanism of *in vitro* bulblet growth in more detail. This should eventually result in procedures that significantly increase the size of lily bulblets and subsequently improve the performance of *in vitro* bulblets after planting in soil.

Materials and Methods

Standard tissue culture conditions

Field-grown bulbs (circumference 18-20 cm) of *Lilium cv. Santander* and *Stargazer* were harvested, cold-treated to break dormancy and stored at -1.0°C until use as described (Aguettaz et al., 1990). Scales were surface-sterilized for 30 min in 1% (w/v) NaClO, rinsed for 1, 3, and 10 min with sterile water, then stored in sterile water (on average for 1-2 h) until use. Two explants of 7 × 7 mm were cut from the scales and placed with the abaxial side on 15 ml medium in plastic culture tubes (3.5 cm diameter). The medium was composed of macro- and microelements (Murashige and Skoog, 1962), 30 g l^{-1} sucrose, 0.4 mg l^{-1} thiamin, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar), and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). The explants were cultured at 25°C and 30 μmol m^{-2} sec^{-1} (Philips TL 33) for 16 h per day.

Different types of explants

Three different types of explants (scale, leaf and petiole) were excised from Santander *in vitro* plantlets, and then cultured for 11 weeks on standard medium supplemented with 2 mg/l NAA and 2 mg/l BA (benzyl adenine) at 25°C and light (30 μmol m^{-2} sec^{-1}; Philips TL 33) for 16 h per day. After 11 weeks, the fresh weight (FW) of lily bulblets and the regeneration percentage were scored. Three Santander and Stargazer scale explant sizes (6x6, 6x12 and 6x18 mm) were cultured on standard

medium at 25°C and 30 μmol m^{-2} sec^{-1} (Philips TL 33) for 16 h per day. After 11 weeks the FW of lily bulblets was measured**.**

To examine the role of lily scales during culture *in vitro*, we cultured 11-week old Santander and Stargazer bulblets fully detached from scale explants and bulblets from which a large part but not all of the original scale explant had been cut off, on standard medium at 25°C and 30 μmol m^{-2} sec^{-1} (Philips TL 33) for 16 h per day. After 6 weeks, the FW of the bulblets was measured.

Explants excised from different positions of Santander and Stargazer scales (see Fig. 1) were cultured on standard medium and kept at 25°C and 30 μmol m^{-2} sec^{-1} (Philips TL 33) for 16 h per day. After 11 weeks the FW of the regenerated bulblets was measured.

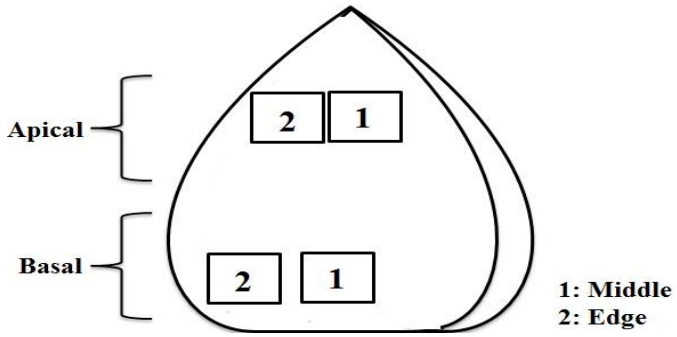

Fig. 1. Schematic drawing of the position of basal, apical, middle and edge explants on a lily bulb scale.

Histological observations

Scale explants of Santander and Stargazer were fixed in 5% glutaraldehyde in phosphate buffer, pH 6.8, and rinsed in the same buffer. They were then dehydrated in an ethanol series and embedded in Spurr's resin. Transversal sections were made on a Sorvall MT 5000 microtome mounted on glass slides, and stained with Toluidin blue to visualize the vascular bundles and with Lugol's IKI solution to visualize starch. The sections were observed with an Axiophot light microscope (Zeiss, Oberkochen, Germany) and images were taken with an AxionCam ERc5S digital camera (Zeiss). The photos were analysed with ImageJ 1.4 software to calculate the starch granule area

in both scale explants in the freshly cut scale explants and after 12 weeks growth *in vitro*.

Statistics

The data were scored 6 and 11 weeks after culturing scale explants in different experiments. The means are the average of 30-100 measurements per treatment. In the figures, the means are shown ± SE. The means were evaluated with a t-test.

Results

The growth of lily bulblets regenerated from various types of explants

The regeneration percentage of scale explants was 88%. In leaf and petiole explants, a much lower regeneration percentage was achieved, *viz.*, 12% and 18%, respectively (Fig. 2a). Scale explants produced bigger and heavier bulblets weighing 51 mg/bulblet compared to leaf and petiole explants weighing 33 and 38 mg/bulblet, respectively (Fig. 2b).

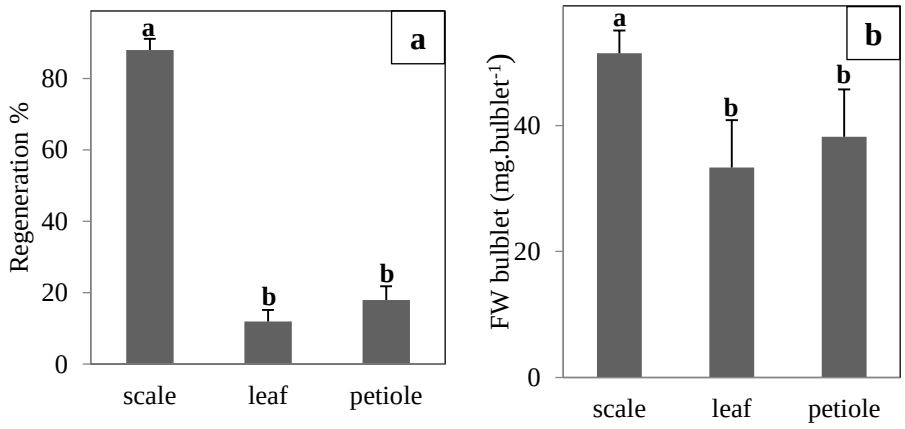

Fig. 2. Regeneration percentage (a); and FW of lily bulblets regenerated on scale, leaf and petiole explants *in vitro* (b) in Santander.

The FW of bulblets increased with the size of scale explants. The FW of bulblets regenerated on 6x18 mm scale explants was 66 mg/bulblet and the FWs of bulblets regenerated on 6x6 mm and 6x12 mm scale explants were 49 mg/bulblet and 53

mg/bulblet, respectively (Fig. 3a). Thus, bigger scale explants improved lily bulblet growth by 26% compared to smaller scale explants.

To study the positive effect of the scale explants in more detail, we excised 11 week-old lily bulblets from scale explants leaving a small piece of scale attached to the bulblets or removing the original scale explant completely. They were cultured on standard medium for 6 more weeks. The FW of lily bulblets with a small piece of explant was 400 mg/bulblet and the FW of lily bulblets from which the original scale

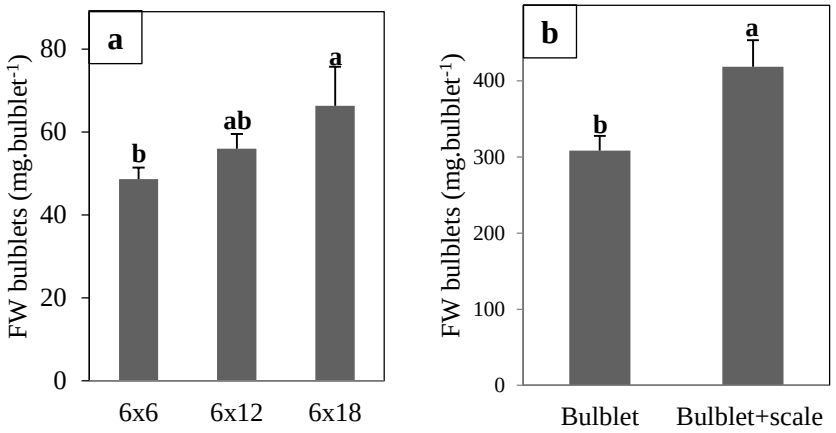

Fig. 3. Effect of the size of the explants on FW of regenerated bulblets (a); Effect of the presence of scale explants on growth of excised bulblets (b) in Santander.

explant had been removed completely was 300 mg/bulblet (Fig. 3b). The initial average FW of 11-week old bulblets at the beginning of each experiment was 100 mg/bulbet for both explant types. In Stargazer, both a large explant and subculturing bulblets leaving a small piece of scale explant attached also improved the growth of bulblets (Data not shown).

We also examined the effect of the original position of the explant on the scale. The various types of explants are shown in Fig. 1. Explants excised from the middle of a scale produced bigger bulblets compared to the explants cut from the edge (ca. 40-50%) and explants cut from the basal part of a scale produced bigger bulblets compared to the explants cut from the apical portion (*ca.* 40-50%, Fig. 4a and b) in both Santander and Stargazer.

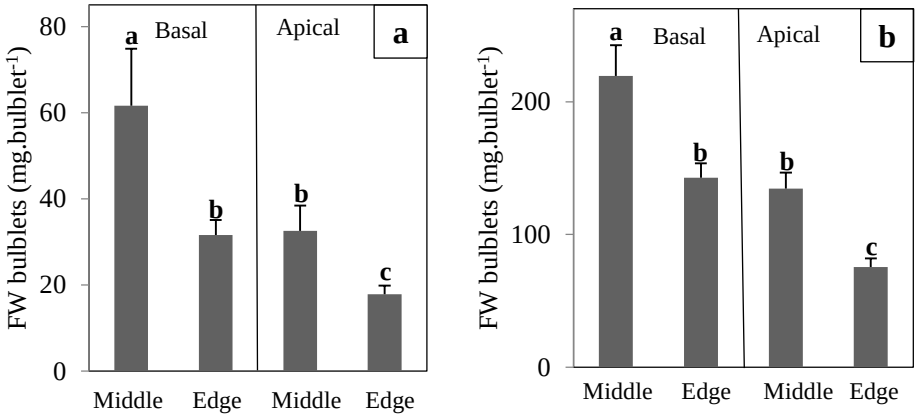

Fig. 4. The effect of scale explant position either basal (middle and edge) or apical (middle and edge) on lily bulblet growth *in vitro*; Santander (a), Stargazer (b).

Contribution of starch to bulblet growth in relation to scale explants position (basal and apical)

Increased growth of the bulblets on basal scale explants compared to apical scale explants raised the question how much carbohydrate (mainly starch) reserve is present in these two types of scale explant tissues at the beginning and at the end of the growth period. Since we were especially interested in the distribution of starch in the tissue, we determined starch by visualizing starch grains in a microscopic examination and evaluating starch content by measuring the area covered by starch granules. The surface area covered with starch granules was 44% and 31% in basal scale explant and 19% and 15% in the apical scale explants at the beginning of the tissue culture period in Stargazer and Santander, respectively (Fig. 5a and c and Fig. 6a and b). The surface area covered with starch granules was 9% and 8% in basal scale explants and 5% and 7% in the apical scale explants at the end of the tissue culture period in Stargazer and Santander, respectively (Fig. 5b and d and Fig. 6a and b). The surface area covered with starch granules decreased 79% and 74% in basal and 74% and 55% in apical explants after 12 weeks in Stargazer and Santander, respectively. In general, the

surface area covered with starch granules in the basal scale explants was *ca*. 2 times larger than in the apical scale explants.

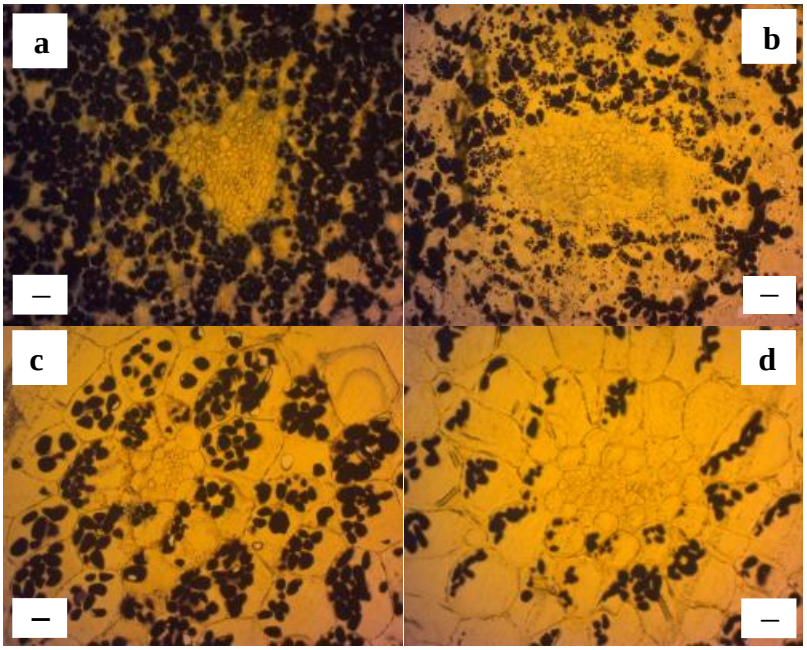

Fig. 5. Distribution of starch in scale explants in relation to their position on bulb scale a) Freshly cut basal explants; b) Basal explants after 12 weeks cultured *in vitro*; c) Freshly cut apical explants; d) Apical explants after 12 weeks cultured *in vitro*. Bar (—) = 5 µm.

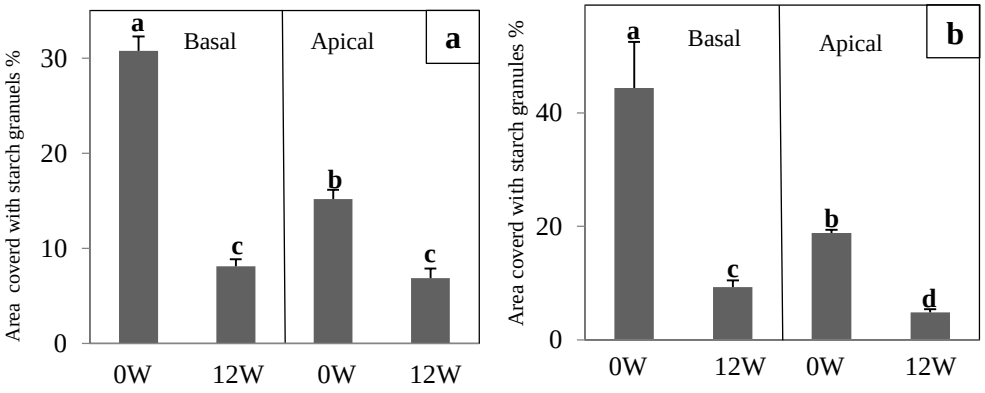

Fig. 6. Surface area covered with starch granules in basal and apical scale explants at the start of culture (0 weeks) and after 12 weeks of culture in Santander (a) and Stargazer (b).

Vascular bundle numbers and size increase during lily bulblets growth in both basal and apical scale explants

In both types of scale explants (0 week and 12 weeks old), vascular bundles were histologically analysed. In freshly cut scale explants, the number of vascular bundles in basal scale explants (6 vascular bundles) was higher than apical explants (3.3 vascular bundles). After 12 weeks, the number of vascular bundles increased in basal and apical scale explants to 8 and 4 vascular bundles, respectively. The area of vascular bundles after 12 weeks was two to three times larger than in freshly cut scale explants in both basal and apical scale explants (Fig. 7 and Fig. 8).

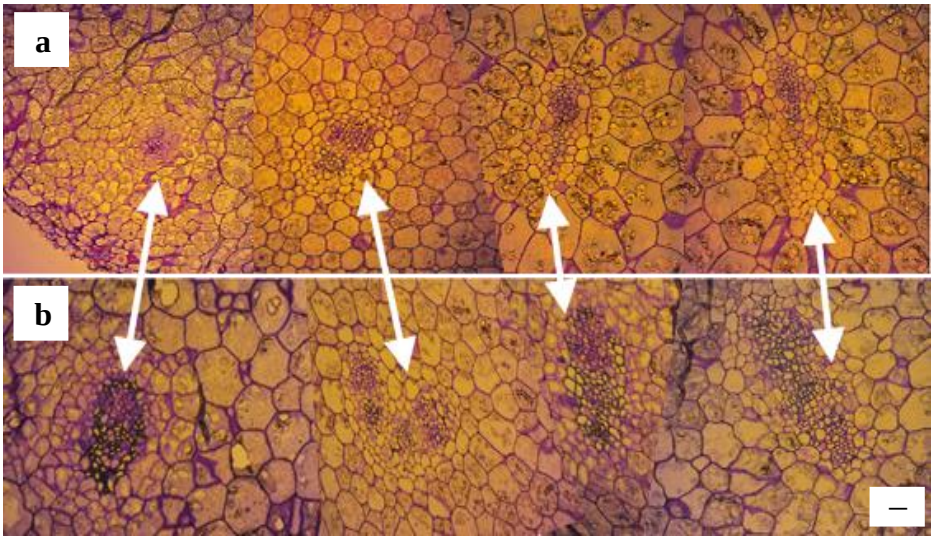

Fig. 7. Increase in the number and the size of vascular bundles in edge basal scale explants; a) vascular tissue in freshly cut edge basal explants of Stargazer; b) the vascular bundles number and size after 12 weeks in the same scale explants; Bar (—) = 25 µm in a, b.

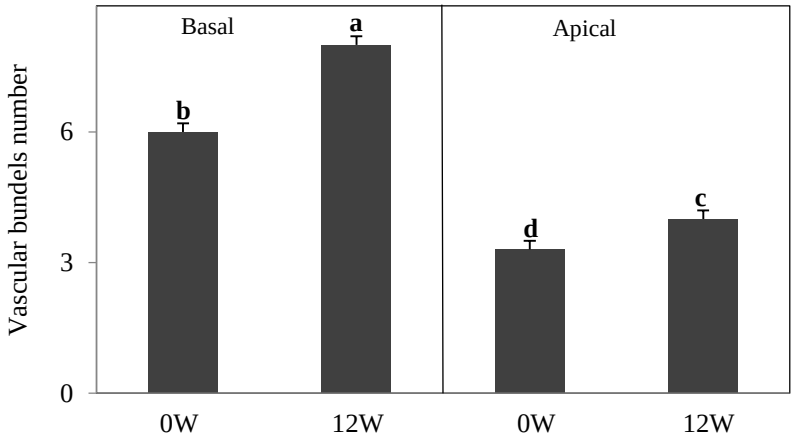

Fig. 8. Vascular bundles number in edge basal and apical lily scale explants at 0 and 12 weeks.

Discussion

The growth of bulblets on different explant types (scale, leaf and petiole)

In lily, regeneration occurs from most tissues, like flower organs, stem nodes, leaves, roots and bulb scales (Gupta et al., 1978; Stimart and Ascher, 1981; Van Aartrijk and Blom-Barnhoorn, 1981; Takayama and Misawa, 1983; Niimi, 1984; Nhut et al., 2001a; Nhut et al., 2001b; Bacchetta et al., 2003). Scale explants are superior explants for micropropagation of lily compared to leaf and petiole explants. The regeneration percentage of leaf and petiole explants was very low compared to scale explants and the FW of bulblets regenerated on the scale explants was higher compared to leaf and petiole explants. The reason why scale explants perform better may be that bulb tissue is more resistant to stress so also to stress related to the transfer to *in vitro* conditions. Moreover, the scale explants contain lots of reserves so that the excised explants depend less on the medium and on transport from the medium.

Previous research showed that large explants regenerated larger bulblets than small explants. A large explant has a large contact area with the medium. Therefore, large explants can take up more nutrients from the medium. The explants act as a sink (growing organ) and as a source (nutrient source for the growing bulblets) simultaneously (Langens-Gerrits et al., 1996b). The bigger explants have more storage materials and vascular bundles to provide the new bulblets with nutrients. Bulblets that

have regenerated from large explants are often adult and the ones from small explants usually juvenile. These bulblets with a different ontogenetic age sprout with a stem or with a rosette, respectively (Langens-Gerrits et al., 2003a). Adult bulblets grow much faster after planting probably because the stem carries many leaves, much more than present in a rosette (Langens-Gerrits et al., 2003a). In other plants, rose (Marcelis-van Acker and Scholten, 1995) and mung bean (Gulati and Jaiwal, 1992) also bigger organs were produced on bigger explants.

The presence of the scale explants promotes the growth of lily bulblets *in vitro*

In lily tissue culture, regenerating bulblets use both carbohydrates broken down from starch in the explants as well as sucrose from the medium to grow (Langens-Gerrits et al., 2003b). The presence of a small piece of scale explant still attached to the bulblets also improved the growth of excised lily bulblets and reveals a major role of the scale explants possibly as some kind of pump. But the starch in the explant also seems to play a role indicated by the breakdown of the starch reserves during 12 weeks of culture. Measurement of starch degradation after 12 weeks of culture of scale explants showed that *ca*. 21% and 26% of starch granules were still available in basal and *ca.* 26% and 45% in apical scale explants in Stargazer and Santander, respectively. In addition, measurement of starch granules in scale explants cultured on medium without sucrose after 12 weeks showed that most starch granules were degraded during bulblet regeneration on medium without sucrose. The largest sink activity (µg sucrose taken up from the medium per mg FW) was found in scale explant and it was constant during tissue culture but sink activity decreased in bulblets regenerated *in vitro* with time (Langens-Gerrits et al., 2003b). These results confirm the major role of the scale explants on the growth of lily bulblets *in vitro*.

Effect of position of the scale explants on lily bulblets growth *in vitro* and visualization of starch granules and vascular bundles in basal and apical scale explants

The position of scale explants influenced the regenerating bulblets growth. As shown in Fig. 4, basal and middle explants regenerated bigger bulblets compared to

apical and edge explants. In the basal part of lily bulb scale, cells are younger, so the basal scale explants are more vigorous to regenerate bulblets than the apical part. Previous research did find that the lower part of a bulb scale was most suitable for multiple shoot differentiation and rapid growth of bulblets for *in vitro* propagation of lily (Long et al., 2003). In tulip, basal scale explants produced more callus lumps and shoots compared to apical scale explants (Koster, 1993). The FW of bulblets, bulblet regeneration percentage and the number of bulblets regenerated *in vitro* was higher on basal scale explants of hyacinth compared to apical scale explants (Pierik and Woets, 1970).

Sucrose taken up in the explants is mainly recovered at the basal side of the explants, where regeneration occurs (Langens-Gerrits et al., 2003b). When lilies are propagated *ex vitro* by keeping complete scales in a moist environment, starch mobilization proceeds from the apical to the basal region of bulb scales (Miller, 1989). The increased bulb growth on the basal explants reflects the higher contribution of explant reserves during bulblet growth *in vitro*. The results of starch granules visualization in basal and apical scale explants indicated that in the basal scale explants more starch granules occur compared to the apical scale explants. In the basal explants, the area of degraded starch granules during lily bulblets growth was *ca.*2 fold higher than in apical scale explants. In field grown lily bulbs, the carbohydrate as well as biomass content of the scales decreased after planting and the degradation of the reserves stored in the outer bulb scale was higher compared to the inner bulb scale (Addai and Scott, 2011). The presence of more starch grains is probably not sufficient to improve bulblet growth when the transport system of nutrients would be the same. We observed that in basal scale explants the number of vascular bundles is almost twice the number than in apical scale explants.

The middle explants in both basal and apical lily scale bulbs include the main vascular bundles and have therefore more and wider vascular bundles. In addition, the explants taken from the middle part are thicker compared to the edge explants. The average FW of middle scale explants and edge explants of the same size (7x7mm) was 250 mg/explant and 150 mg/explant, respectively. The middle scale explants have therefore more storage reserves compared to the edge scale explants. Both types of

explants grow considerably during the tissue culture period. After 11 weeks, the average FWs of middle scale explants and edge scale explants were 1300 mg/scale and 1400 mg/scale, respectively. This result also shows that bulblets regenerated on edge scale explants used less storage material from the scale explants compared to the middle scale explant because in the beginning even the FW of middle scale explant was higher than edge scale explants but at the end the difference between the FW of edge scale explants and middle scale explants was not statistically significant. This indicates that the vascular bundles play an important role during lily bulblet growth *in vitro* due to translocation of medium component and transport of scale storage organs to the bulblets regenerated *in vitro.*

Development of the vascular bundle number and size during bulblet regeneration *in vitro* occurred in both basal and apical scale explants. In plants, development of new vascular tissues enables regeneration of the plant and its adaptation to interruptions and changes in the environment (Aloni, 1987) so increase in number and the size of vascular bundles during lily bulblets growth *in vitro* is relevant to the growth of lily scale explants and may be a logic response of scale explants to changes in the growth situations to adapt to the new growth conditions. In tulip, development of scale explant vascular bundles to meristematic centre occurred after 10 days and callus lumps at the upper side of scale explants appearing after 30 days originated from meristematic cells of vascular bundles (Koster, 1993).

Conclusion

This study shows that characteristics of the scale explants play a major role in the growth of bulblets that regenerate from the scales. Scale explants are the best explants for micropropagation of lily bulblets *in vitro*. The size of scale explants at the beginning of tissue culture is a useful tool to produce bigger bulblets during lily *in vitro* micropropagation. It is related to more starch granules that are degraded during the period of bulblet regeneration and more and wider vascular bundles to transfer the nutrients to the bulblets regenerated *in vitro*. In addition, the significant difference between the growth of lily bulblets on basal and apical scale explants also indicated

that the amount of starch granules and the intensity of vascular bundles play a major role in lily bulblet growth *in vitro*.

Chapter 4

The effect of abiotic stresses on lily bulblet growth *in vitro*

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Abstract

We examined effects of different abiotic stresses on the growth of lily plantlets *in vitro.* Twelve week old lily plantlets responded with an increase in the fresh weight of bulblets by 20 % to hot air treatment (HAT). At a younger age the HAT stress had no significant effect. To assess the most effective temperature to apply HAT stress a range of temperatures (32 °C - 47°C) was examined. The most effective temperature to apply HAT was 44°C which led to an increase of lily bulblet growth by 30%. The effective duration of HAT and hot water treatment (HWT) was 2h and 3h respectively. A moderate HAT pre-treatment (38°C) protected the lily bulblets against severe HAT (47°C). In non-pretreated bulblets which were exposed for 2h to severe HAT stress the survival percentage of plantlets decreased to 10% and 25% in cultivars Stargazer and Santander, respectively. In Stargazer at 6h drought stress the growth of scale explants decreased by 35%, but the growth of lily bulblets, roots and leaves increased by 40%, 177% and 34%. In Santander drought stress decreased the growth of lily bulblets and scale explants and improved the growth of roots and leaves with increase of the duration of drought stress from 0 to 10 h. In Santander, a 10 h drought stress reduced the growth of bulblets and scale explants by 30% and increased the growth of roots and leaves by 42% and 14%. Finally, the application of anaerobiosis as stress led to increase of the growth of lily bulblets (after an 8h treatment) by 65% and 32% in Santander and Stargazer, respectively**.**

Introduction

Lily is one of the most valuable ornamental plants. The flowers are popular because of their color and shape. The Netherlands produces 76% of the lily bulbs worldwide (Qu, 2014). Lily was one of the first floricultural plants which was propagated in tissue culture. In 1957, the first report on bulblet regeneration of lily from scale explants *in vitro* was published (Robb, 1957). A scheme for commercial production was published in 1983 (Takayama and Misawa, 1983).

The size of the bulblets produced *in vitro* has a strong effect on plant performance after planting. Studies with direct field planting of bulblets produced *in vitro* have shown that small bulblets emerge slower, less uniform and to a smaller percentage (Langens-Gerrits et al., 2000; Lian et al., 2003). It also was observed that bulblets of plantlets growing in soil, show a much faster growth than bulblets growing *in vitro* (De Klerk et al., 1992).

Plants have developed several mechanisms for protection during adverse periods. One of the main protective mechanisms is dormancy accompanied by storage organ formation. In this way, plants do not grow and do not produce (vulnerable) new tissue during stressful conditions. Regrowth when conditions are suitable is supported by mobilization of nutrients from the storage organ. Plants under stressful conditions tend to increase biomass in subterranean organs to protect against stress (Puijalon et al., 2008). For instance, in bulbous crops moderate heat stress can improve the formation of bulbs and roots of chives (Fölster and Krug, 1977). In *Alstroemeria* moderate stresses enhance the growth of rhizomes *in vitro* as a protective reaction (Pumisutapon et al., 2012).

In the present study, the growth of lily bulblets regenerated *in vitro* was monitored after applying different moderate stresses. We show that moderate abiotic stresses such as heat, drought and anaerobosis improved the growth of lily bulblets *in vitro*.

Materials and Methods

Standard tissue culture conditions

Field-grown bulbs (circumference 18-20 cm) of *Lilium cv.* Santander and Stargazer were harvested, cold-treated to break dormancy and stored at -1.0°C until use. The procedure was according to (Aguettaz et al., 1990). Scales were surface-sterilized for 30 min in 1% (w/v) NaClO, rinsed for 1, 3, and 10 min with sterile water and after that stored until use in sterile water (on average for 1-2 h). Two explants of 7 × 7 mm were cut from the scales and placed with the abaxial side on 15 ml medium in plastic culture tubes (3.5 cm diameter). The medium was composed of macro- and microelements (Murashige and Skoog, 1962), 30 g l^{-1} sucrose, 0.4 mg l^{-1} thiamin, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar), and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). The explants were cultured at 25°C and 30 μmol m^{-2} sec^{-1} (Philips TL 33) for 16 h per day. After 12 weeks unless indicated otherwise, different abiotic stresses were administered and the explants were allowed to growth for 6 weeks more.

Heat treatments

To determine the effect of the temperature during the hot air treatment (HAT), for each treatment 30 explants of 12 week old bulblets were placed in an incubator at different temperatures: 32 °C, 35 °C, 38°C, 41°C, 44°C, and 47°C for 1 hour after which the explants were cultured for 6 weeks more at a temperature of 25 °C in a growth chamber. To assess the effect of applying HAT for different times, 30 explants for each treatment were incubated in an incubator at 44°C for 1, 2, 3, 4, and 5 hours after which the explants were incubated in a growth chamber for 6 weeks more. For examination of the effect of bulblet age, 30 containers with explants of 6, 8, 10 and 12 week old bulblets were placed in an incubator (Labcon LTIM 10). The temperature was kept at 38 °C for 1.5 hour. After that, the explants were cultured in a growth chamber at 25 °C for 6 weeks.

To find out whether moderate stresses protect the lily bulblets against severe stresses, lily bulblets were pre-treated at 38 °C for 1 or 2 hours and then after 4 hours treated with severe HAT at 47 °C for 1 or 2 hours. Next, the explants were kept for 6 weeks in a growth chamber at 25 °C. Finally, survival and growth were determined.

For hot-water treatment (HWT), 25 explants were placed in a sterile beaker (200ml) with sterile water (100ml) and incubated for 1.5 or 3 hours in a water bath (Lauda Bath Circulator C12) at 38 °C after which the explants were cultured on MS standard medium in small plastic containers with 15 ml medium and kept at 25 °C for 6 weeks.

Application of other stresses

The effects of anaerobiosis and drought stress on bulblet growth were investigated. For anaerobiosis the explants were submerged in sterile water (15 explants per treatment) for 0, 2, 4, 8, 24 and 72 hours in the dark at 25 °C followed by culture on standard MS medium for 6 more weeks at 25 °C. Drought stress was given by keeping the explants on dry filter paper in the laminar air flow cabinet at room temperature for 0, 2, 6 and 10 hours after which the explants were cultured for six more weeks on standard medium at 25 °C.

Statistics

All the data were scored 6 weeks after applying the different abiotic stresses. The fresh weight (FW) of lily bulblets, scale explants, roots and leaves were determined. The means are the average of 15-30 measurements per treatment. In the figures, the means are shown ± SE. The differences were evaluated with a *t*-test.

Results

High temperature stress enhances lily bulblet growth *in vitro*

To examine the most suitable temperature, containers with lily bulblets of Stargazer regenerated for 12 weeks under standard conditions were transferred to a range of temperatures (25-47°C). After 1 h HAT they were kept for 6 weeks at 25 °C in a growth chamber. The growth of lily bulblets increased significantly at 44 °C (with 30% compared to the control) (Fig. 1).

Then, the optimal duration of HAT at 44 °C was determined. As shown in Fig. 2a, the growth of lily bulblets increased (40%) in 1 and 2 h HAT at 44 °C, but at the longer durations of 3, 4 and 5 h HAT, the bulblet growth decreased dramatically.

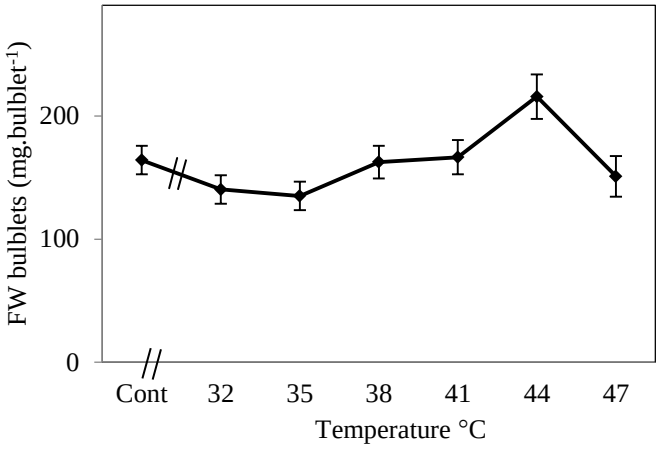

Fig. 1. The effect of 1h temperature treatment (HAT) on bulblet growth during the following 6 weeks. The weight that is shown is after the 6 weeks of extra growth. Cont is control (ambient temperature, 21 ºC).

For the hot water treatment (HWT), two types of explants, bulblets and bulblets still attached to a small piece of scale explant, were submerged in hot (38 °C) water for 0, 1.5 and 3 h, then kept for 6 weeks more at 25 °C in a growth chamber and then evaluated with respect to growth. The results showed that the lily bulblet growth increased in both explant types and the highest bulblet growth of about 40% occurred in 3 h HWT in explants attached to a small piece of scale explant (Fig. 2b).

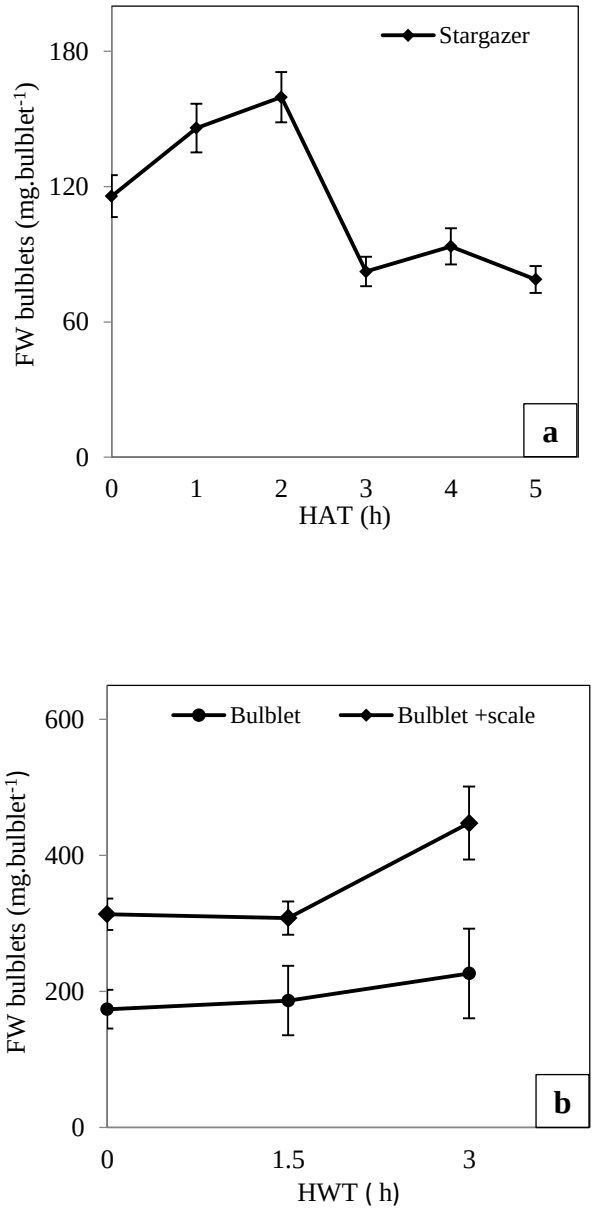

Fig. 2. Effect of duration of HAT (a) and HWT (b) on bulblet growth during the following 6 weeks. The weight that is shown is after the 6 weeks of extra growth.

To find out whether lily bulblets of different ages respond in the same or in a different way, 1.5 h HAT at 38 °C was applied at 6, 8, 10 and 12 weeks after the start of culture of the scale explants on standard medium (Fig. 3). Bulblet growth increased ca. 20% when the HAT was applied to scale explants at 12 weeks of age compared to the control. At age 6, 8 or 10 weeks, HAT did not show a positive effect on the growth of lily bulblets.

Moderate HAT protects against severe HAT

Figures 1-3 show that the moderate heat stresses have an effect in the long run (a period of weeks). We examined whether they also had an effect in the short run (a period of hours). A moderate HAT does protect lily from a severe HAT. The results (Fig. 4a and b and Fig. 5) show that fresh weight of lily bulblets in both cultivars decreased significantly when no pre-treatment was applied. In Stargazer, a 1h HAT pre-treatment at 38 °C followed by 1h severe HAT at 47°C increased the fresh weight of bulblets compared to a higher duration of severe HAT. Longer severe HAT in Stargazer had a negative effect on the bulblet growth. In Stargazer, the highest fresh weight of bulblets occurred by applying a 2h moderate HAT without severe HAT. In Santander, the highest bulblet fresh weight was found by applying 2h moderate HAT followed by 2h severe HAT. The data showed that severe HAT has a small positive effect on the bulblet growth of Santander bulblets.

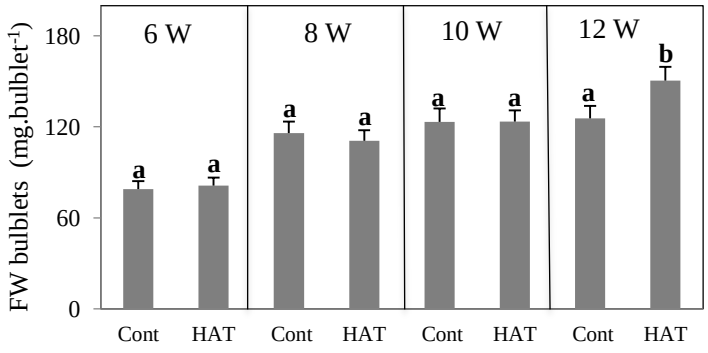

Fig. 3. Effect of 1h HAT at cultures of different ages on bulblet growth during the next 6 weeks. The weight that is shown is after the 6 weeks of extra growth.

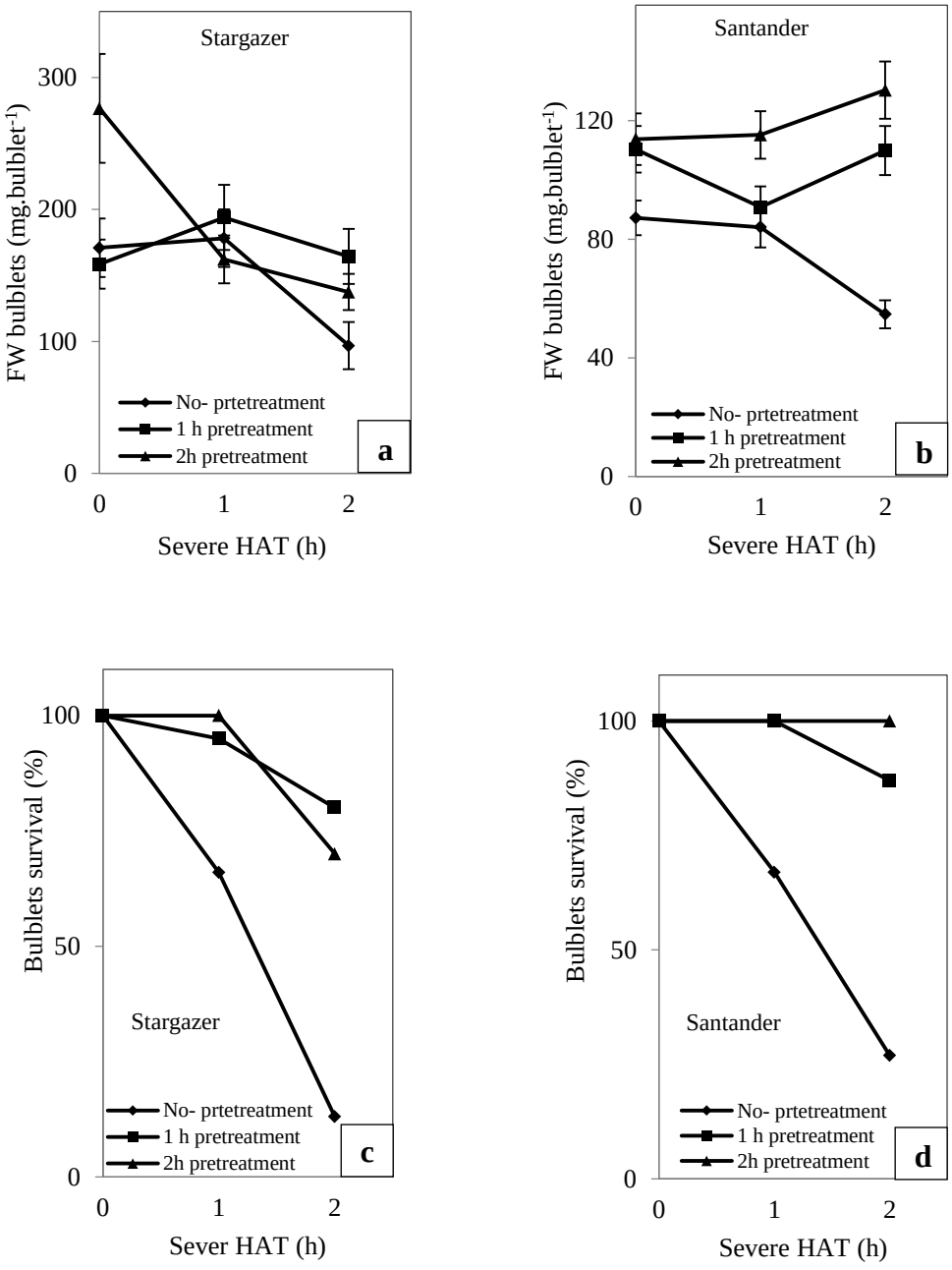

Fig. 4. Effect of 38 °C HAT pre-treatment followed by 47 °C severe HAT on bulblet growth during the next 6 weeks of cvs. Stargazer (a) and Santander (b) and bulblet survival percentage in Stargazer(c) and Santander (d). The weight that is shown is after the 6 weeks of extra growth.

The survival percentage of bulblets after 2h severe HAT was reduced to 10% - 25% when no pre-treatment with moderate HAT was given (Fig. 4c and d; Fig. 5). The pre-treatments rescued in both cultivars a large percentage of bulblets.

Fig. 5. Effect of moderate stress administered before a severe stress on cv. Santander. a) Control; b) only 2h severe stress; c) 1h moderate pre-stress+2h severe stress; d) 2h moderate pre-stress+2h severe stress.

Other stresses

We also examined other stresses. Drought stress was applied for 0, 2, 6 and 10 hours on 12 weeks old bulblets under sterile conditions. The results indicated that the fresh weight of bulblets in Santander reduced when drought stress was applied (Fig. 6). In contrast, the fresh weight of Stargazer increased significantly when drought stress was given. For 2 and 10 h drought stress there was no significant difference, but the increase at 6 h drought stress (40%) was significant.

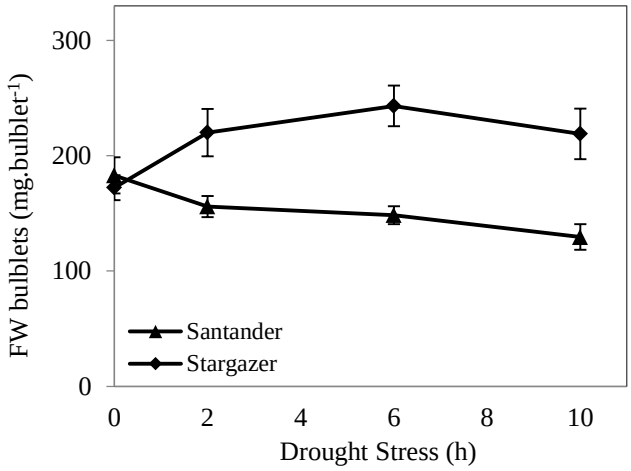

Fig. 6. Effect of drought stress on bulblet growth during 6 weeks after the stress in Stargazer and Santander. The weight that is shown is after the 6 weeks of extra growth.

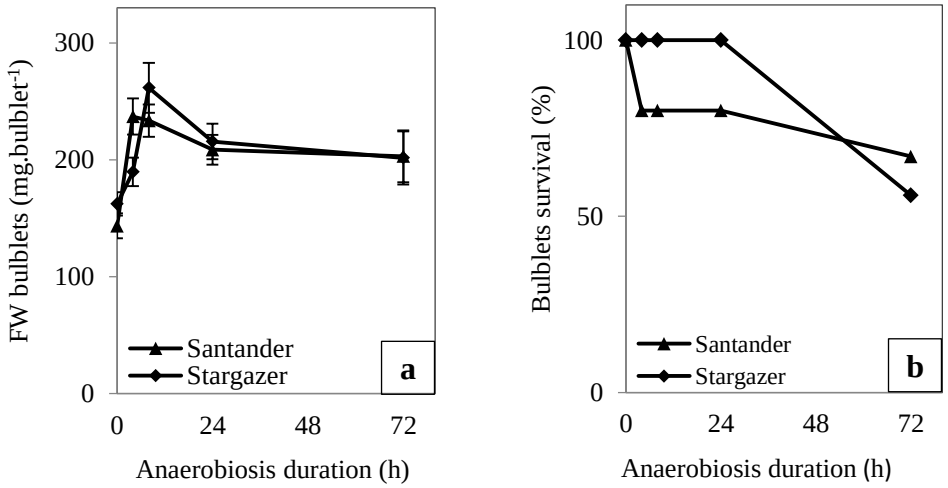

Fig. 7. Effect of anaerobiosis on bulblets growth (a) and bulblets survival (b) during 6 weeks after the stress. The weight that is shown is after the 6 weeks of extra growth.

Different durations of anaerobiosis treatment (0h, 4h, 8h, 24h and 72 h) were applied on the explants and the fresh weight of lily bulblets increased after shorter duration anaerobiosis treatment compared with the control in both cultivars (Fig. 7a).

The highest amount of fresh weight was measured after 4h and 8h anaerobiosis treatment with an increase of fresh weight of 65% and 32% in *cvs.* Santander and Stargazer respectively. Longer durations of anaerobiosis had a negative effect on fresh weight of both cultivars and reduced the survival percentage in both cultivars too (Fig. 7b).

Discussion

Improvement of lily bulblet growth

Plants maturity accelerates under heat stress conditions to overcome unprecedented stress (Nagarajan and Nagarajan, 2009) and in many plants leads to mass allocation of carbohydrates to the storage organs (Chapin et al., 1990) to survive and regrow after stressful conditions. The growth of lily bulblets increased after a moderate stress treatment (HAT, HWT, drought stress and anaerobiosis stress) *in vitro*. In a previous report (Pumisutapon et al., 2012) the growth of *Alstroemeria* rhizomes increased after moderate abiotic stress *in vitro*. On the other hand, the growth of potato tubers and onions reduced after applying heat stress and salt stress respectively (Ewing, 1981) and (Chang and Randle, 2004).

The growth of bulblets increased by almost 40% in HWT (Fig. 2). The lily bulblets were exposed to both high temperature and anaerobiosis because the bulbs were submerged in hot water for a long time and better heat conduction in water and additional moderate stresses by anaerobiosis may lead to the improved growth (Jackson, 1985) and (Dolferus et al., 2003). Moreover, the small pieces of explant attached to the bulblets increased the bulblet growth under HWT stress compared to bulblets without scale explant. It seems that the scale explant is a favorable nutrient source compared to the medium during growth period after applying HWT.

In moderate HAT, lily bulblets growth increased at 44 °C and lower temperatures had no impact on the bulblet growth. In addition, longer duration of HAT had a negative effect on lily bulblet growth and development.

Plants under moderate stresses may be primed to respond better or endure longer under future severe stresses. This has been illustrated in *Alstroemeria* (Pumisutapon et al., 2012) where a moderate HWT stress protected *Alstroemeria* against severe HWT.

In lily both cultivars showed a positive effect of pre-treatment HAT compared to non-pretreated bulblets. The moderate HAT stress may activate some thermo-tolerance genes to protect against severe HAT. A heat shock transcription factor gene called *LlHsfA2*, has been reported to play an important role in heat signalling pathway in lily and it has been demonstrated that overexpression of *LlHsfA2* can increase the thermo-tolerance of transgenic *Arabidopsis* plants (Xin et al., 2010). This observation is strengthened by the fact that *LlHSFA1* isolated from lily leaves and overexpressed in transgenic *Arabidopsis* gave similar results (Gong et al., 2014).

Only Stargazer has shown a positive response to drought stress on bulblet growth. On the other hand, anaerobiosis increased bulblet growth in both cultivars. We assume that increasing the growth of lily bulblets *in vitro* under different abiotic stresses trace back to the natural response of plants under adverse conditions. Under these stressful conditions plants tend to increase biomass in subterranean organs as a way to protect against future stresses.

Conclusions

Moderate stress increases the growth of lily bulblets probably due to a protective mechanism against abiotic stress. This method is a useful way to stimulate lily bulblet growth *in vitro* to achieve bigger bulblets during tissue culture period. The optimal conditions as assessed in two different lily cultivars are anareobiosis and HAT to increase the growth of lily bulblets. Depending on the variety, a pre-treatment with moderate HAT stress (38°C) seems to be a valuable way to enhance the response to more severe HAT stress (47°C).

Chapter 5

CO_2 starvation *in vitro* is lethal at heterotrophic conditions

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Abstract

We examined the effect of CO_2 starvation on growth *in vitro* with 3% sucrose or without sucrose in the nutrient medium. The experiments were carried out with lily bulblets and *Arabidopsis* seedlings. CO_2 removal from the headspace of tissue culture containers by a 20% KOH solution reduced the growth of all organs of a regenerating bulblet (bulblets, leaves, roots) and growth of the original scale explant. Reduced growth was observed both in the absence of sucrose in the medium and when 3% sucrose had been added to the nutrient medium. Growth reduction was higher in the absence of sucrose (depending on the organ 33-79%) but still considerable with 3% sucrose in the medium (29-33%). Similar results for growth reduction were observed in excised 11-week old lily plantlets growing *in vitro* with or without sucrose under CO_2 starvation or 'normal' conditions. CO_2 removal from the headspace also decreased growth of *Arabidopsis* seedlings *in vitro* on medium with 3% sucrose or without sucrose. F_v/F_m dropped both in lily and in *Arabidopsis* under CO_2 starvation. In lily, F_v/F_m decreased from 0.69 to 0.60 and in *Arabidopsis* from 0.76 to 0.62. F_v/F_m of *ex vitro* growing lily and *Arabidopsis* was 0.77 and 0.79, respectively. Occurrence of ROS was examined in *Arabidopsis* seedlings by staining with nitroblue tetrazolium (NBT). ROS was virtually absent in *ex vitro* growing seedlings and very abundant in seedlings growing *in vitro* under CO_2 starvation. Seedlings grown under normal tissue culture conditions showed an intermediate level of ROS.

Introduction

The microenvironment in tissue culture containers is very different from the environment in which plants normally grow. Apart from the medium (containing high doses of organic and inorganic nutrients and plant hormones), and the low light intensity, the tissue culture environment is characterized by an unusual atmosphere. The relative humidity is extremely high (continuously very close to 100%), and the headspace contains high levels of organic gases such as ethylene and highly fluctuating CO_2 and O_2 at temporary very low and very high levels (Kozai 1991). The CO_2 concentration in an air-tight vessel containing green plantlets is often lower than the CO_2 compensation point during most of the photoperiod, viz., less than 100 µmol.1^{-1}, which is much lower than the normal atmospheric CO_2 concentration of 400 µl. l^{-1}. Even in loosely capped vessels or vessels capped with gas permeable film, the concentration is often lower than 200 µmol. l^{-1} (Kozai 1991). During the dark period, CO_2 increases up to 3000 to 9000 µl l^{-1} (Fujiwara et al. 1987).

The low CO_2 concentration in the vessels and the low light intensity limit photosynthesis. Poor photosynthesis *in vitro* may also be caused by the high sugar concentration in the medium (Kozai 1991; Desjardins et al. 1995). A reduction of the sucrose concentration enhances net photosynthesis, e.g., in *Rosa multiflora* (Capellades et al. 1991), and rain tree (Mosaleeyanon et al. 2004). However, addition of sugar is necessary for successful tissue culture: in spite of the increase of photosynthesis, use of medium without sucrose reduces the growth of plantlets dramatically compared to medium with 3% sucrose. Therefore, medium without sucrose is not useful for the plant tissue culture industry.

A very low CO_2 level during exposure to light is deleterious for plants due to the lack of electron acceptors in the photosynthetic electron transport chain (Durchan et al. 2001). In the course of CO_2 starvation, all endogenous electron acceptors become reduced and oxygen is the main available electron acceptor. Oxygen can serve as electron acceptor in the Mehler reaction (Mehler 1951; Schreiber and Neubauer 1990). The products of the Mehler reaction such as superoxide, hydroxyl radicals and hydrogen peroxide (ROS) are toxic, and attack vulnerable macromolecules.

Reactive oxygen species (ROS) are by-products in various metabolic pathways in plants. In stressful conditions, ROS rapidly increase (oxidative burst) and these are toxic for plants (Apel and Hirt 2004; Ramel et al. 2009). ROS is removed by different antioxidative defense components.

In the present study we examined whether the low CO_2 in tissue culture is damaging for plant tissue. We first studied plants cultured in tissue culture in an atmosphere from which almost all CO_2 was removed by a 20% KOH solution. We used *Arabidopsis* seedlings and lily bulblets regenerating from scale explants as two contrasting tissue culture systems. We observed strongly reduced growth both in lily and *Arabidopsis* as a result of CO_2-poor conditions. We used chlorophyll fluorescence for investigating the damage to the photosynthesis of plants. As a comparison, we also used chlorophyll fluorescence to evaluate any potential damage inflicted during a standard tissue culture cycle. Chlorophyll fluorescence (PSII efficiency) is a simple and widespread method (Baker 2008) to establish photosynthesis performance. The ratio of variable (F_v) to maximal (F_m) chlorophyll fluorescence (F_v/F_m) in photosystem II of the photosynthesis apparatus is believed to be an index for maximum photon yield. F_v/F_m has been used to assess the efficiency of electron transport and/or damage to photosystem II (Maxwell and Johnson 2000; Leipner et al. 2001).

We observed a significant reduction of chlorophyll fluorescence during tissue culture when CO_2 was removed indicating severe damage leading to an observed reduced growth. Under normal tissue culture conditions, F_v/F_m was also reduced albeit much less.

Materials and Methods

Lily bulblet regeneration from scale explants

Field-grown bulbs (circumference18-20 cm) of *Lilium* cv. Santander were harvested, cold-treated to break dormancy and stored at -1.0 °C until use. The procedure for lily was according to (Aguettaz et al. 1990). Scales were surface-sterilized for 30 min in 1% (w/v) NaClO, rinsed for 1, 3, and 10 min with sterile water and stored until use in sterile water (on average for 1-2 h). Two explants of 7 × 7 mm were cut from the scales and placed with the abaxial side on 30 ml medium in plastic

culture tubes (6.5 cm diameter). The medium (MS) was composed of macro- and microelements (Murashige and Skoog 1962), 30 g l^{-1} sucrose or without sucrose, 0.4 mg l^{-1} thiamin, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar), and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). All chemicals were obtained from Duchefa, Haarlem, the Netherlands. The scale explants were cultured for 12 weeks at 25°C and 30 μmol m^{-2} sec^{-1} light for 16 h per day (Philips TL 33).

Growth of lily plantlets

11-week old lily plantlets regenerating from scales under standard conditions (with sucrose) were excised from the scales and cultured on medium with MS macro- and microelements (Murashige and Skoog 1962), 30 g l^{-1} sucrose or without sucrose, 0.4 mg l^{-1} thiamin, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar), and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). The plantlets were cultured for 6 weeks at 25°C and 30 μmol m^{-2} sec^{-1} light for 16 h per day (Philips TL 33).

***Arabidopsis* seedling growth in medium with 3% sucrose or without sucrose**

Arabidopsis thaliana (Col-0) seeds were sterilized with 70% (v/v) ethanol for 1 min and in 2% (w/v) sodium hypochlorite for 15 min. They were subsequently rinsed three times for 10 min with sterilized distilled water. Sterile seeds were transferred to a Petri dish with half-strength Murashige and Skoog (MS) basal salt mixture including vitamins (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose and solidified with 7 g l^{-1} agar (Microagar). Seeds were stratified in the dark for 3 d at 4 °C and after that cultured in a growth chamber with 16 h light/8 h dark (30 μmol m^{-2} s^{-1}, Philips TL33) at 21 °C. After 7 d, the seedlings were transferred to fresh medium with 30 g l^{-1} sucrose or without sucrose then incubated for two weeks in a growth chamber with 16 h light/8 h dark (30 μmol m^{-2} s^{-1}, Philips TL33) at 21 °C.

Removal of CO_2 from the headspace

A small vial was placed on the medium next to the explants (Fig. 1 and Fig. 5). This vial contained 3 ml 20% KOH and a piece of filter paper standing vertically to increase the contact surface between the KOH solution and the atmosphere in the

headspace. CO_2 was removed from the headspace according to the reaction KOH + CO_2 -> $KHCO_3$. The KOH solution also reduced the relative humidity (Solomon 1951) and a saturated KCl solution was used as control for this. Both the 20% KOH and saturated KCl resulted in *ca.* 85% RH.

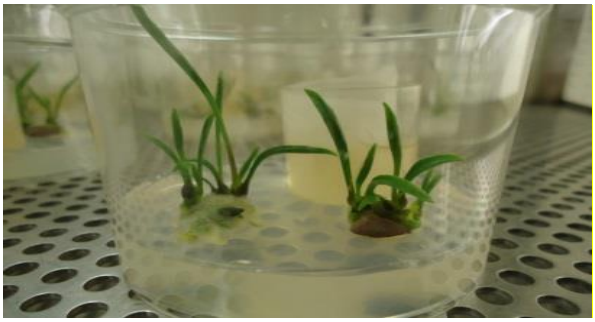

Fig. 1. Container with lily scale explants on a nutrient medium with 3% sucrose and a vial with KOH-solution to remove CO_2.

Measurement of maximum PSII quantum yield (F_v/F_m)

Lily plantlets and *Arabidopsis* seedlings were cultured *ex vitro* in 8-cm pots filled with potting soil or in tissue culture containers as described above in control and CO_2-poor conditions in medium with 3% sucrose. After *in vitro* growth (12 weeks for lily and 3 weeks for *Arabidopsis*), the leaves were used for measurements in a chlorophyll fluorescence imaging system (FluorCam, Photon System Instruments, Brno, Czech Republic). Intact leaves still attached to the plants in tissue culture containers or in pots were dark-adapted for 20 min. After dark adaptation intact plants (*in vitro* or *ex vitro*) were immediately used to measure maximum quantum efficiency of photosystem II (F_v/F_m). In the FluorCam imaging system, an 512 x 512 pixel, CCD camera was used to record fluorescence images. F_v/F_m was calculated using a custom-made protocol. Images were recorded during short measuring flashes in darkness. These flashes were provided by two panels each containing 345 orange light emitting diodes. At the end of the short flashes a 250 W halogen lamp produced a one second duration saturating light pulse. The saturating light pulse had an intensity of 2,500 mmol m^{-2} s^{-1} that results in a transitory saturation of photochemistry and reduction of

primary quinone acceptor of photosystem II. After reaching steady state fluorescence, two successive series of fluorescence data were digitized and averaged, one during short measuring flashes in darkness (F_0), and the other (F_m) during the saturating light flash. From these two images, F_v was calculated by the expression $F_v=F_m-F_0$. The F_v/F_m was calculated using the ratio $(F_m-F_0)/F_m$. The average values, and standard deviation of F_v/F_m per image were calculated by using version 5 of FluorCam software (Genty et al. 1989; Aliniaeifard et al. 2014; Aliniaeifard and Van Meeteren 2014).

Visualization of superoxide radicals

Superoxide radicals were detected by staining 3-week old *Arabidopsis* seedlings with nitroblue tetrazolium (NBT) solution according to (Van Den Dries et al. 2013) with minor modifications. *Arabidopsis* seedlings were cultured *ex vitro* in 8-cm pots filled with potting soil or in tissue culture containers as described above in control and CO_2-poor condition in medium with 3% sucrose. Seedlings with roots were transferred into a 0.1% (w/v) NBT solution containing 50 mM phosphate buffer (pH 7.8) and 10 mM NaN_3. The seedlings were vacuum-infiltrated for 5 min and kept for 45 min in the dark at room temperature. Stained seedlings were then bleached in acetic acid – ethanol 80% (1/4) (v/v) at 100 °C for 30 min. Seedlings were then stored in 50% ethanol until photographs were taken.

Statistics

Fresh weight (FW) was scored after 12 weeks (scale explant culture; FW bulblets, FW leaves, FW roots and FW scale explant), after 6 weeks (lily plantlet culture; FW bulblets, FW leaves and FW roots), and after 3 weeks (*Arabidopsis* seedling culture; FW shoots and FW roots). Thirty explants were used for each observation. In the figures, the means are shown ± SE. The means were evaluated with a *t*-test.

Results

Effect of CO_2 removal during bulblet regeneration from scale explants

The presence of sucrose in the nutrient medium increased the growth of explants strongly compared to medium without sucrose. In medium with 3% sucrose, removal of CO_2 caused a significant reduction in lily growth (Fig. 2). Bulblet growth decreased by 33% compared to the control (Fig. 2a), the leaf fresh weight by 68% (Fig. 2b) and the root FW by 70% (Fig. 2c). The FW of scale explants was 1356 and 285 mg/scale under control and CO_2-poor condition respectively. The growth of scale explants decreased by 79% compared to the control (Fig. 2d). Removal of CO_2 significantly reduced growth of lily bulblets grown in the absence of sucrose by 23% (Fig. 2a), the FW of leaves by 21% (Fig. 2b), the FW of roots by 34% (Fig. 2c) and the FW of scale explants by 25 % (Fig. 2d) compared with the control.

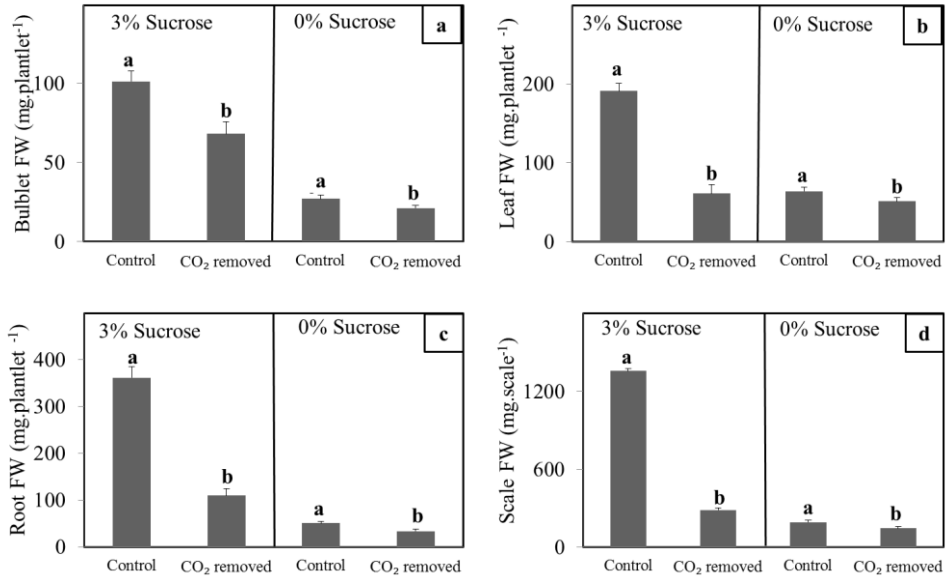

Fig. 2. Effect of CO_2 removal from tissue culture containers on lily growth: bulblets (a), leaves (b), roots (c) and scale explants (d) during bulblet regeneration *in vitro* in medium with 3% sucrose and without sucrose.

Effect of CO_2 removal during culture *in vitro* of excised 11-week old lily plantlets

11-Week old plantlets regenerated at standard conditions, were excised from the scales and cultured for another 6 weeks on nutrient medium with 3% sucrose or without sucrose. There were significant differences between FW of bulblets, leaves and roots between medium with 3% sucrose and without sucrose. In medium with 3%

sucrose, the FW of bulblets was 309 and 219 mg/bulblet in control and CO_2-poor condition respectively, with a start weight of bulblets of 174 mg/bulblet for both control and CO_2-poor treatment. CO_2-poor condition caused significant reduction in bulblet growth by 29% (Fig. 3a), leaves growth by 52 % (Fig. 3b) and root growth by 31% (Fig. 3c) in medium with 3% sucrose. CO_2-poor treatment reduced profoundly the lily bulblet growth by 162%, leaf growth by 60% and root growth by 92% in medium without sucrose.

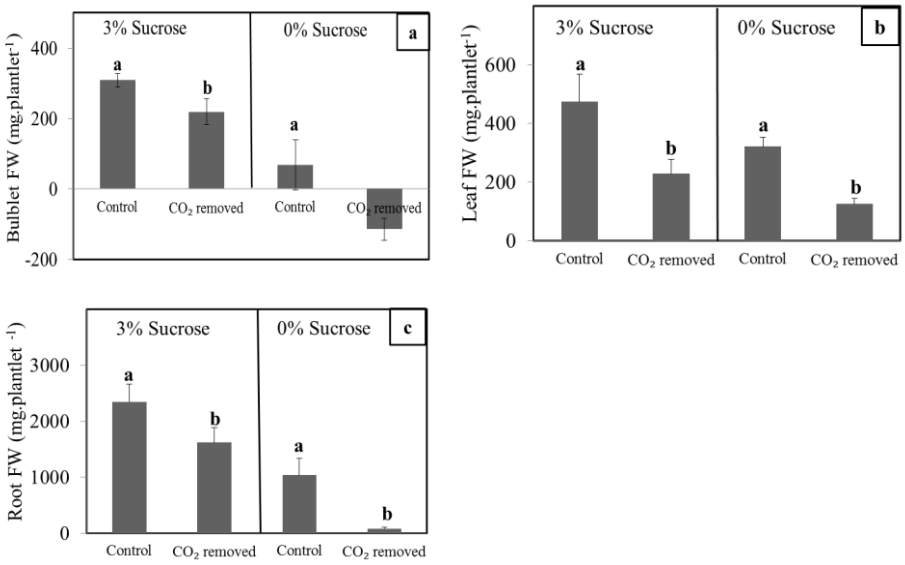

Fig. 3. Effect of CO_2 removal from tissue culture containers on 11-week old lily plantlets growth: bulblet (a), leaves (b) and roots (c) in medium with 3% sucrose and without sucrose.

Effect of CO_2 removal during *in vitro* culture of *Arabidopsis* seedlings

Removal of CO_2 from the headspace of the tissue culture containers seems related with very general physiological processes. To examine whether effects of CO_2 removal from the headspace are general, growth of *Arabidopsis* seedlings in medium with 3% sucrose or without sucrose with or without removal of CO_2 were examined. In medium with 3% sucrose, the FW of *Arabidopsis* shoots was reduced by 50 % (Fig. 4a) and roots by 65% (Fig. 4b) in CO_2-poor conditions. In medium without sucrose, *Arabidopsis* shoot growth was reduced by 78% (Fig. 4a) and root growth by 65% (Fig. 4b) in CO_2-poor conditions. With the exception of seedlings grown with sucrose and

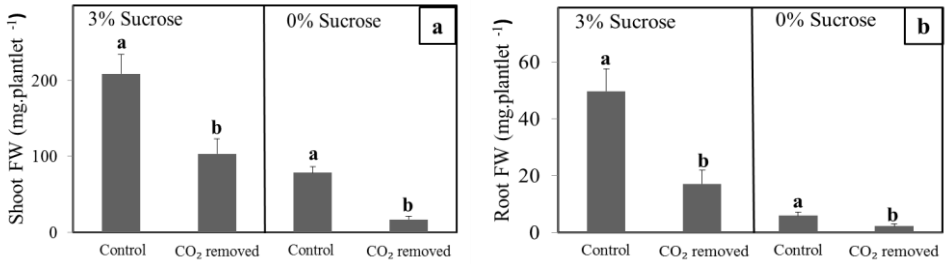

Fig. 4. Effect of CO_2 removal on growth of *Arabidopsis* seedlings in medium with 3% sucrose and without sucrose.

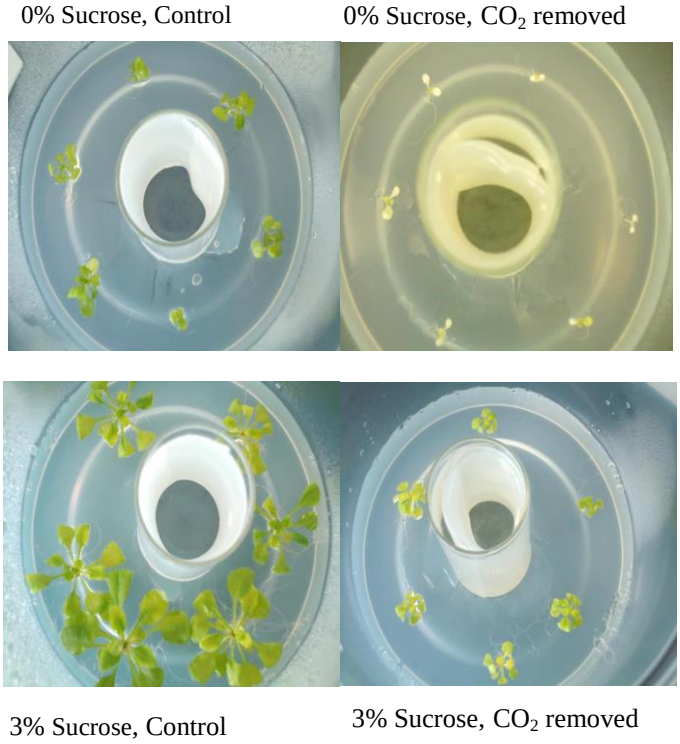

Fig. 5. *Arabidopsis* seedlings cultured for 7 days on medium without (0%) or with sucrose (3%). A small vial with 3 ml 20% KOH was added to remove CO_2 from the headspace. As a control for the drop of relative humidity (RH) by 20% KOH solution, also a small vial with saturated KCl was added.

without removal of CO_2, the seedlings were in a very poor condition and died soon after the period of observation (Fig. 5). It should also be noted that seedlings bleached in CO_2-poor condition.

Effect of CO_2 removal on F_v/F_m of lily and *Arabidopsis* and ROS detection

To investigate the effect of CO_2 removal on photosynthetic performance, F_v/F_m was measured in lily plantlets and *Arabidopsis* seedlings grown on medium with 3% sucrose. In general, higher F_v/F_m was observed in *Arabidopsis* compared to lily (Fig. 6). The results showed that in both plant species, *in vitro*-generated plants had lower F_v/F_m compared with the F_v/F_m of *ex vitro* (in soil) plants (Fig. 6). Furthermore, in *in vitro*-generated lily and *Arabidopsis* plants, F_v/F_m was significantly decreased in CO_2-poor condition in comparison with its value in control *ex vitro* and *in vitro* plants (Fig. 6 and Fig. 7 c, f).

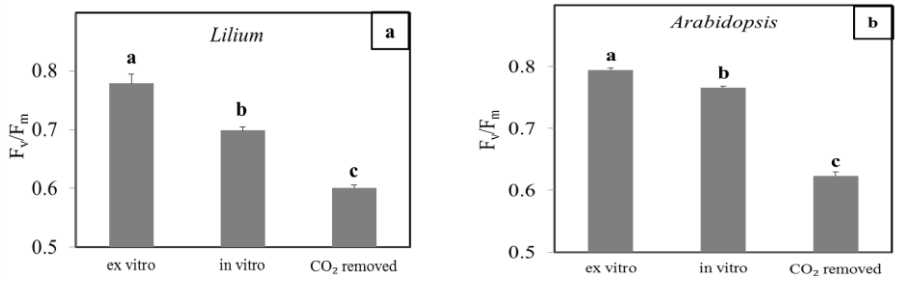

Fig. 6. F_v/F_m measurement in lily and *Arabidopsis* (*ex-vitro*, *in vitro* and CO_2 removed).

Arabidopsis seedlings grown *in vitro* on medium with 3% sucrose with and without CO_2 were stained with NBT. When CO_2 had been removed, the seedlings became deeply blue showing the abundant formation of ROS (Fig. 8c) whereas the in vitro condition with CO_2 shows only little blue staining. *Arabidopsis* seedlings grown in *ex vitro* conditions remained colourless (Fig. 8a).

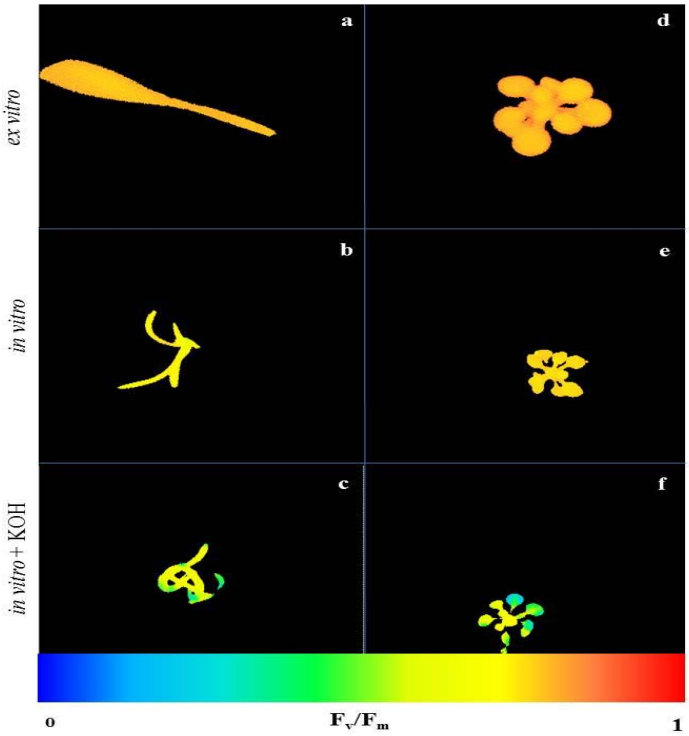

Fig. 7. Image of F_v/F_m a) lily grown *ex vitro*, b) lily grown *in vitro*, c) lily grown in CO_2-poor condition and d) *Arabidopsis* grown *ex vitro*, e) *Arabidopsis* grown *in vitro* and f) *Arabidopsis* grown in CO_2-poor condition.

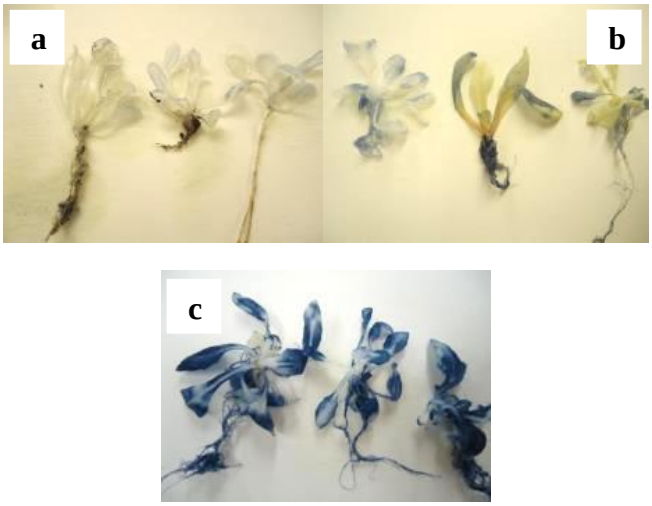

Fig. 8. *Arabidopsis* seedlings stained with NBT a) *Arabidopsis grown ex vitro*, b) *Arabidopsis* grown in control condition and c) *Arabidopsis* grown in CO_2-poor condition.

Discussion

In tissue culture, sucrose is applied via the nutrient medium to support growth. Tissue culture is usually carried out in the light and the plant material is chlorophyllous. Under these conditions, photosynthesis is inevitable although at a reduced rate. Indeed at standard tissue culture conditions the characteristic curves for CO_2 and O_2 during a daily regime of light and dark have been observed: high CO_2 / low O_2 levels in the dark and low CO_2 / high O_2 levels during the day (Debergh et al. 1992). The extent to which photosynthesis contributes to growth is not known but seems to be limited during standard tissue culture conditions since growth is very much reduced when no sucrose is added (e.g. see in the present article Fig. 2 and Fig. 4).

We removed most CO_2 from the headspace by a 20% KOH solution. Just as when omitting sucrose, we observed a very large effect on growth. This is, however, most likely due to a detrimental side-effect of CO_2 withdrawal, the abundant formation of ROS (see next section). In this way, the contribution of photosynthesis to growth *in vitro* cannot be determined. Other ways, in particular feeding $^{14}CO_2$ may be a solution, but this was not investigated in our study.

An important finding in this paper is the huge detrimental effect of low CO_2 on the plantlets (Figs. 2, 4 and 5). This can be traced back to the formation of ROS (Fig. 8c). Abundant formation of ROS was measured in *Arabidopsis* seedlings grown on medium with 3% sucrose in CO_2-poor condition. Chlorophyll remains active in absorbing energy from light also when CO_2 is very low. But when CO_2 is not available as an electron acceptor in the photosynthetic electron transport chain, other electron acceptors become reduced, in particular oxygen, resulting in toxic, activated oxygen species (Durchan et al. 2001).

A reduced electron transport flux through the photosynthetic electron chain and subsequently ROS formation has also been reported in the presence of high sugar in medium even under normal condition of light (Desjardins et al. 2009). For high sucrose concentration contradictory effects on photosynthesis activity and ROS formation have been reported. Several authors report that high sucrose concentrations in medium is deleterious for photosynthetic activity (Ehness et al. 1997; Serret et al. 1997). However, sucrose has also been reported to stimulate photosynthesis in *in vitro* plantlets (Tichá et

al. 1998; Fila et al. 1998). ROS formation is also stimulated (Takahashi and Murata 2008) or decreased (Couée et al. 2006) by sugar.

When CO_2 was removed from the headspace by a 20% KOH solution, *Arabidopsis* leaves showed significant bleaching and F_v/F_m dropped strongly. In addition, growth declined drastically as well. In lily, bleaching was not so strong (not shown) but F_v/F_m dropped even more than in *Arabidopsis* (Fig. 6). Growth reduction in lily was more severe in leaves than in bulblets. This may be because the leaves are the site with abundant chlorophyll and bulblets contain much less chlorophyll (Fig. 2, compare also *Arabidopsis* in Fig. 4), so most damage might be done in the leaves.

When there was no KOH solution in the tissue culture container, we still observed a significant reduction in the F_v/F_m of the leaves (Fig. 6). This may be related to a very low level of CO_2 brought about by photosynthesis (see Introduction). This decrease of the F_v/F_m indicates damage to the tissue that may lead to a general reduction in growth. ROS detected in *Arabidopsis* seedlings in common tissue culture condition compared with *ex vitro* plantlet showed that the *in vitro* plantlets in general suffered from *in vitro* culture condition (Fig. 8 a and b).

A study on gene expression in *in vitro* and *ex vitro* tomato leaf tissues showed that stress related genes and ROS scavenging enzymes were up regulated in *in vitro* tomato plants. Superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) enzymes eliminate toxic superoxide and hydrogen peroxide by conversion to water. The expression of APX and GR were higher in tomato leaf tissues *in vitro*, compared to *ex vitro*, which leads to adaptation and acclimatization to *in vitro* conditions (Dubuc et al. 2009). Up regulation of ROS scavenging enzymes in tomato leaf tissues grown *in vitro* showed that plants build up mechanisms to overcome stressful *in vitro* conditions by removing detrimental free radicals. But in severe stressful conditions like the CO_2-poor conditions we applied in *Arabidopsis* the abundant amount of ROS formation (Fig. 8c) severely damaged the growth of *Arabidopsis* seedlings (Fig. 4a and b) and after a few weeks the plants died.

Monitoring of lipid peroxidation and antioxidant activation during acclimatization processes of gerbera plantlets *in vitro* indicated that the amount of MDA (malondialdehyde), a marker for lipid peroxidation which is an effect of

oxidative damage, and hydrogen peroxide (H_2O_2) were higher at 0 day compared with 25 days after transfer from *in vitro* to *ex vitro* conditions. The activity of APX, SOD, GR and CAT (catalase) dropped between 0 to25 days after transfer of gerbera plantlets from tissue culture containers to soil (Chakrabarty and Datta 2008). As we found ROS in *Arabidopsis* grown in normal tissue culture conditions (Fig. 8b) and lower F_v/F_m in both lily and *Arabidopsis* grown *in vitro* compared with *ex vitro* (Fig. 6), we assume that a reduction in both ROS and antioxidant enzymes activity after the transfer of gerbera plantlets from *in vitro* to *ex vitro* conditions mostly traced back to removal of *in vitro* stressful conditions and an adaptation of gerbera plantlets to *ex vitro* conditions.

Higher growth of *ex vitro* plants compared with *in vitro* plantlets was observed in lily (De Klerk et al. 1992) and tulip (Hulscher et al. 1992). So, the lower growth of *in vitro* plantlets might be related to the damaging effect of ROS on the growth of *in vitro* plantlets compared with *ex vitro* plants.

Conclusion

In conclusion, photosynthesis is important for the growth of lily and *Arabidopsis in vitro*. Photosynthesis supplies the plant tissue with sucrose, but it is not known whether this contribution is substantial or marginal. A second major effect of photosynthesis is prevention of the formation of ROS by elicitated chlorophyll. This effect is very substantial.

Chapter 6

General Discussion

Flowering geophytes are among the most preferred ornamental plants because of their aesthetic features, fragrance and suitability to be used as cut flowers, pot plants and garden plants. The Netherlands produces approximately 65% of world flower bulbs. The Dutch flower bulb sector exports 76% of bulb flowers in the world market. In the Netherlands, 5% (23590 ha) of arable land (Buschman, 2004) is covered by bulbous crops for bulb production, mainly tulip (11440 ha), lily (5220), daffodil (1680 ha), hyacinths (1480 ha), gladiolus (1110 ha), crocus (470 ha) and iris (250 ha) (Rabobank, 2015).

Just as most floricultural crops, geophytes are propagated vegetatively. This is conventionally done in the field but as propagation is not fast, many years in the field are required. Such long periods in the field necessitates extensive chemical protection. From an environmental point of view, the production of flower bulbs on arable lands is quite challenging as surface and underground water have been contaminated by the use of pesticides and fertilizers (Jansma et al., 2000). This is accepted less and less by society. Moreover, a large percentage of the plants still harbours pathogenic microorganisms. An illustration of the emerging problems with environmental pollution is conventional propagation of tulip. Conventional propagation of tulip is the Achilles heel of this symbol of The Netherlands due to the low rate of *in vitro* shoot and bulblet formation and slow growth of the *in vitro* bulblets (Van Rossum, 1997).

Production of pathogen-free starting materials, rapid introduction of cultivars bred for resistance, and reduction of the period of growth in the field, make tissue culture the desirable alternative tool for propagation of ornamental geophytes instead of conventional propagation methods. Because of their robustness, storage organs (bulbs, tubers) are the ideal propagules for geophytes. Thus, for micropropagation of geophytes, *in vitro* storage organ formation is one of the focal points of research.

Production of high quality *in vitro* bulblets (bigger and uniform size) has a direct influence on the performance in the field and leads to a reduction of the field growth period in the flower bulb production processes. With 88% of world tulip bulbs, and 77% of world lily bulbs being produced in the Netherlands (Buschman, 2004), a study on the improvement of geophytes tissue culture is extremely important from an economical and environmental point of view for The Netherland. We have selected lily

as the second economically most valuable ornamental geophyte as experimental model for elucidation and improvement of bulblet growth *in vitro*.

Lily breeding and tissue culture

Lily breeding is a lengthy process and introduction of a newly bred lily cultivar on the market takes up to 15 years. First, selection of the best clone takes several years: from seed to flowering plant takes about three years so that the first selection for flower properties is only possible several years after the initial crosses have been made. Then, several years are needed to evaluate quantitative properties like yield or resistance to diseases. After selection of the best clones, production of a sufficient number of bulbs takes again several years due to the low speed of the available vegetative propagation methods (natural propagation and scaling). Micropropagation considerably shortens this propagation period and is nowadays used in most breeding programs. Starting with one bulb, it is possible to produce a large number of genetically identical bulblets *in vitro* in a relatively short time. Because of the high propagation rates *in vitro*, newly bred cultivars can be introduced on the market nowadays within 7-8 years and tissue culture thus plays an essential role in the rapid expansion of the lily assortment (Langens-Gerrits, 2003).

The main constraints in conventional propagation of lilies include the insufficient availability of healthy, disease-free planting material, and slow multiplication rates. One of the best and most prolific vegetative propagation methods for lilies *in vitro* is scale culture (Varshney et al., 2001; Bahr and Compton, 2004). On the bulb scale explants, bulblets regenerate and after 8–10 weeks these bulblets can be used for further propagation. Individual scales of bulblets, or parts thereof, are used as explant for the next propagation cycle and a cycle can be repeated every 8–10 weeks. Using tissue culture, from one large bulb, about one million small bulblets can be obtained in 2 years (Langens-Gerrits, 2003). From a commercial point of view, micropropagation is a fast and disease-free propagation method for lily. Bulblets and other storage organs produced *in vitro* have properties that make them preferable propagules. They can be easily handled, transported and stored and they do not require an extensive acclimatization procedure after transfer to soil (Thakur et al., 2006).

The size of the bulblets produced *in vitro* has a strong effect on performance after planting. Studies with direct planting of bulblets produced *in vitro* have shown that small bulblets emerge slower, less uniform and to a smaller percentage (Lian et al., 2003). When bulblets are sufficiently large (>300 mg), they increasingly sprout with a stem instead of a rosette (Langens-Gerrits et al., 2003a). Performance after planting depends on the physiology of the bulblets at the time of planting. Obviously, these physiological characteristics have been formed during the culture *in vitro*. Three main factors are relevant: bulblet weight, ontogenetic age (maturation) and dormancy status. It is desirable to produce large bulblets *in vitro* as they show a larger increase of fresh weight per bulblet after planting than small ones (Langens-Gerrits et al., 1996a).

The aim of this thesis was to improve the growth of lily bulblets as a model bulbous crop by studying the basic and applied aspects of the following main topics:

- The increase of bulblet growth under a more stringent aseptic condition (Chapter 2),
- The effect of several scale-explant related factors (Chapter 3),
- The increase of bulblet growth by moderate abiotic stresses (Chapter 4) and
- The decrease of bulblet growth by incomplete photosynthesis (Chapter 5).

Approaches to enhance lily bulblet growth *in vitro*

Many plant organs can be used as explants in micropropagation of ornamental geophytes. Aerial segments of plants (leaf, pedicle, sepal, petal, ovary, embryo anther) and undergrounds organs (tuber, bulb, rhizome, corm and tuberous root) were used as explant in tissue culture of geophytes (Kim and De Hertogh, 1997). The most commonly used explant for micropropagation of geophytes are bulb scales (Mirici et al., 2005). The greatest problem in tissue culture of underground organs like bulb scales is the high percentage of contamination, which can result in a significant loss of cultures during the *in vitro* period (Kim and De Hertogh, 1997). Apart from inadequate operating during manipulation in the laminar flow cabinet, poor equipment (e.g., damaged filters in the laminar flow cabinet) and contamination by micro-arthropods (mites and thrips), the biggest source of contamination is the explant itself that is transferred into tissue culture. A wide range of microorganisms (filamentous fungi,

yeasts, bacteria, viruses and viroids) has been detected as contaminants in plant tissue culture (Altan et al., 2010). Underground organs attached to soil may carry higher numbers of inner and outer microorganisms compared with aerial organs. This leads often to a defective sterilization process in the *in vitro* initiation procedure of bulbous crops. All kinds of contaminants, have been causing considerable economical losses in commercial tissue culture laboratories (George, 1993; Reed et al., 1998; Leifert, 2000). Scientists tried to tackle this problem in different ways. The effect of fungicides, antibiotics, pre-treatment with hot water, and sugar-free media were examined (Shields et al., 1984; Langens-Gerrits et al., 1998; Kubota and Tadokoro, 1999). Increase in the growth of explants has been also reported due to side effects of decontamination agents in tissue culture. For instance, application of low concentrations of benomyl (a systemic fungicide which is taken up and translocated by plant cells and organs) as a decontamination agent in medium enhanced the growth of roots and shoots in tissue culture of *Asparagus officinalis* (Yang, 1976).

In **Chapter 2** we used NaClO at a very low concentration (0.03%) for additional disinfection. This concentration was effective and is also reported to be adequate in medical practice (Heling et al., 2001). First, we examined cross-contamination during the sterilization/rinsing procedure. When the scales are rinsed with sterile water for the 2nd and 3rd time, the rinsing water may become heavily contaminated with bacteria when infected scales are still present. This resulted in considerable additional contamination of the explants. We used a simple way (rinsing with 0.03% NaClO instead of water) to reduce this cross-contamination. Bulblet growth increased with 22% by reducing cross-contamination. The second cause for development of contamination in lily scale explants is the open connection between the vascular tissues and the environment when the scales are detached from the mother bulbs. This allows movement of microorganisms into the vascular tissue. Entering is strongly enhanced by the negative hydrostatic pressure in the tissue, which results in sucking up of fluids into the xylem just after excision. These fluids often contain microorganisms. We used again 0.03% NaClO to control these contaminants (C**hapter 2).** Bulblet growth increased with 17% by reducing this type of contamination. These effective measures in lily are most probably also valid for other species.

Storage organ formation is a process controlled by interacting environmental, developmental and genetic factors. Studies on different stages of storage organ formation, induction, initiation and growth of storage organs (De Hertogh and Le Nard, 1993; Sarkar, 2008), can be considered on the morphological, physiological, biochemical and molecular level (Podwyszynska, 2012). Bulb scales are the main explants used in tissue culture of bulbous crops (Mirici et al., 2005). Many valuable bulbous crops like lily (Varshney et al., 2000; Skorić et al., 2014), amaryllis (Ilczuk et al., 2005), hyacinths (Yi et al., 2002) and daffodils (Santos and Salema, 2000) are propagated via bulb scale explants *in vitro*. The size of lily bulblets produced *in vitro* strongly affects performance after planting. Studies with direct field planting of *in vitro* bulblets have shown that small bulblets emerge slower and less uniform and have a lower sprouting percentage (Lian et al., 2003). After transfer to soil large tulip bulblets regenerated *in vitro* also show better performance compared to small bulblets (Le Nard et al., 1987; Hulscher et al., 1992). *In vitro* lily bulblets in the adult phase sprout with a stem and switch to a reproductive state; on the other hand in the juvenile phase, bulblets sprout with a rosette. Large bulblets are more often in the adult phase compared to small bulblets (Langens-Gerrits et al., 2003a).

In **Chapter 3**, we examined lily bulblet growth and regeneration percentage in different lily explants including, petiole, leaves and scale explants. Scale explants gave a higher bulblet regeneration percentage and the growth of lily bulblets regenerated on scale explants was higher than on the other explants. The reason why scale explants perform better may be that bulb tissue is more resistant to stress so also to stress related to the transfer to *in vitro* conditions. Moreover, the scale explants contain lots of reserves so that the excised explants depend less on the medium and on transport of solutes from the medium. The bigger scale explants produced bigger lily bulblets *in vitro* most probably due to a higher amount of starch granules and a higher vascular bundle intensity.

In this chapter we also showed that the presence of a small piece of scale explant attached to the bulblets improved the growth of excised lily bulblets and reveals a major role for the scale explants possibly as some kind of “pumping” unit. But the starch in the explant also seems to play a role even though the starch reserves are not

exhausted after 11 weeks of culture. The growth of lily bulblets regenerated on basal scale explants was higher compared with apical scale explants. Staining of starch granules in the same area around vascular bundles of basal and apical scale explants at two stages, freshly cut scale explants and after 12 weeks cultured on medium showed that the basal scale explants are covered with more starch granules. In addition, the number and development of vascular bundles during tissue culture period in complete basal scale explant (7x7 mm) was more than apical scale explant in freshly cut scale explants and after 12 weeks cultured on medium. More starch granules and more vascular bundles in basal scale explants probably traced back to higher cellular density due to the smaller size of the cells in the younger part of bulb scales. These results indicated that starch content and vascular bundles are important for an efficient growth of lily bulblets *in vitro*. Thus, keeping a piece of scale explant after bulblet regeneration *in vitro* during sub culturing, using bigger scale explants and using basal and middle parts of bulb scale as scale explant resulted in the production of bigger bulblets. These results may be also applied to *in vitro* micropropagation of other bulbous crops.

Stress conditions (drought, salinity, heat, anaerobiosis) reduce growth, development and productivity in plants. Plants have developed different mechanism to overcome stressful conditions. Tolerance mechanisms are activated by different abiotic stresses and result in the accumulation of different protective low molecular weight compounds (Bohnert et al., 1995) *viz.*, proline, glycine, betaine, polyamine or trehalose, and protective protein, *viz*., heat shock proteins (HSPs; chaperone-function, (Wang et al., 2003). Plants in stressful conditions tend to allocate a high proportion of biomass to below ground biomass (roots and storage organs) compared to above ground (Fritz et al., 2004). Dormancy accompanied by storage organ formation is also one of the main protective mechanisms in plants. In this way, plants do not grow and do not produce (vulnerable) new tissue during stressful conditions. Regrowth when conditions are suitable is supported by mobilization of nutrients from the storage organ. Plants under stressful conditions tend to increase biomass in subterranean organs to protect against stress (Puijalon et al., 2008). For instance, in bulbous crops moderate heat stress can improve the formation of bulbs and roots of chives (Fölster and Krug, 1977). In

Alstroemeria moderate stresses enhance the growth of rhizomes *in vitro* as a protective reaction (Pumisutapon et al., 2012).

In **Chapter 4**, we examined several moderate abiotic stresses (HAT, Hot Air Treatment; HWT, Hot Water Treatment; drought stress and anaerobiosis stress) on lily bulblets growth. The growth of lily bulblets increased after a moderate stress *in vitro*. Moderate HAT and HWT and anaerobiosis increased the growth of lily bulblets. Depending on the cultivar, drought stress also increased the bulblet growth. We examined the effect of HWT on two explant types (bulblets and bulblets attached to a small piece of scale explant) and found that the presence of a piece of scale explant increased the effect of the HWT. We assume that the scale explant has a role in the positive effect of abiotic stresses by accelerating mobilization of the reserves. We also found that moderate abiotic stresses can protect lily against further severe abiotic stresses *in vitro*. Thus, the moderate abiotic stresses are a promising tool to promote the growth of lily bulblets and may be used for other storage organs.

Photosynthesis is the most characteristic physiological process in all green plants. Different components, including photosynthetic pigments, photosystems, the electron transport system and CO_2 reduction pathway are involved in photosynthesis. In general, any damage at any level caused by stresses may reduce the overall photosynthetic capacity of green plants (Ashraf and Harris, 2013). In *ex vitro* conditions, a lot of research has been conducted and described to determine the influence of stresses like drought (Medrano et al., 2002; Dias and Brüggemann, 2010), salt (Aragão et al., 2005; Abdel-Latif, 2008), and high temperature stresses (Wang et al., 2010) on photosynthesis**.**

In general, *in vitro* conditions are not the most favourable situation for plants to grow in. Composition of the medium, limited gas exchange, high humidity, low light, mechanical injuries and wounding are unfavourable elements that plants have to acclimatize to in order to survive and grow. Inhibition of photosynthesis by high sugar concentrations *in vitro* has been reported in several plants. In strawberry, high concentrations of sucrose (3% and 5%) in the medium caused a notable reduction in photosynthetic activity compared with 0% and 1% sucrose in the medium (Hdider and Desjardins, 1994). Further studies showed that sucrose inactivated Rubisco and

increased sugar phosphates, which interacted and inhibited the carboxylation sites (Hdider and Desjardins, 1995). Addition of sucrose in medium also reduced the expression of photosynthetic genes (Jones et al., 1996). Feedback inhibition of photosynthesis induced by the presence of sugar increased the oxidative stress both in chloroplasts and mitochondria. ROS production above a putative threshold caused damage in the presence of high sugar concentrations *in vitro* (Desjardins et al., 2009).

In **Chapter 5**, we applied a severe CO_2-poor condition by removal of CO_2 from the headspace of tissue culture containers by a small vial filled with a 20% KOH solution. The removal of CO_2 was done to investigate the deleterious effect of continuous severe CO_2-poor conditions during lily and *Arabidopsis* growth *in vitro*. Just as when omitting sucrose, we observed a very large effect on growth. This is, however, most likely due to a detrimental side effect of CO_2 withdrawal, that is the abundant formation of ROS. When CO_2 is not available as an electron acceptor in the photosynthetic electron transport chain, other electron acceptors become reduced, in particular oxygen, resulting in toxic, activated oxygen species. Abundant formation of ROS was measured in *Arabidopsis* seedlings grown on medium with 3% sucrose in CO_2-poor condition. Low F_v/F_m and ROS in normally *in vitro* grown *Arabidopsis* and lily compared with *ex vitro* grown plants indicated that *in vitro* is a stressful condition for these plants.

Conclusions and future prospects

In all geophytes, also in the economically prominent ornamental geophytes like tulip, lily, hyacinths, iris and narcissuses, the formation of storage organs *in vitro* is a slow and problematic process. Although micropropagation protocols have been developed for all major geophytes, most are not sufficiently workable in commercial laboratories and a major reason for this is inadequate bulb formation. So research is needed to study and improve *in vitro* storage organ formation. Especially, since the Netherlands is the leader of bulbous crops production in the world, improvement of *in vitro* propagation of geophytes has a direct economic and environmental effect at the national level.

In general, studies on geophytes are difficult, time-consuming and risky due to the lack of physiological, biochemical and molecular knowledge and to the long growth period. *In vitro* storage organ formation is hard due to the long-term storage organ formation in tissue culture, in the case of lily 3-4 months and for tulip 6-8 months, while many of the physiological, biochemical and molecular mechanisms, which are relevant for in vitro growth, are fully unknown. We developed new and valuable knowledge with respect to different aspects of lily bulblet growth *in vitro*, but there is still much need to further investigate the *in vitro* storage organ formation.

We introduced two novel and simple ways to reduce contamination in bulbous crops. We added a diluted NaClO solution as rinsing fluid to avoid cross contamination. We identified negative-hydrostatic-pressure related contamination and reduced it with a diluted NaClO solution. For further investigations, we propose to use other decontaminating components like antibiotics and fungicides specifically to reduce negative hydrostatic pressure related contamination. Penetration of antibiotics or fungicides assisted by the negative hydrostatic pressure into scale explants may reduce genuine endogenous contamination that has entered the bulbs during culture in the field.

We also performed a detailed study about the effect of scale explants on lily bulblets regeneration *in vitro*. We found that scale explants, even after regeneration period, are a valuable tool to improve lily bulblet growth. Starch and vascular bundles also seem to play a major role in regeneration and growth of lily bulblets *in vitro*. We propose to investigate mobilization and degradation of scale explant storage reserves during tissue culture in more detail using labelling with ^{3}H-sucrose and ^{14}C-sucrose. In addition, a study of detailed anatomical changes like vascular bundles development during bulblet regeneration and growth *in vitro* is needed. Scale explants increase fresh weight and even synthesise new starch granules in newly formed tissue like callus, but in bulb scales the fresh weight decreased during scaling. Studies on different morphological and physiological growth patterns between scale explants during bulblet regeneration *in vitro* and scaling (vegetative propagation) may give results to reveal more information about mechanisms of storage organ formation.

We also report the positive effect of abiotic stresses on lily bulblet growth *in vitro*. We propose to examine combined abiotic stress on lily bulblets growth.

Applying abiotic stresses examined in this study to more cultivars of lily and to other geophytes and testing the field performance of treated plants may lead to the identification of specific abiotic stresses to apply in commercial laboratories.

We examined the effect of CO_2-poor conditions on lily and *Arabidopsis* growth *in vitro*. Investigation of CO_2-poor conditions as a severe stressful condition *in vitro* at the molecular level, especially in *Arabidopsis* as a model crop, can be a valuable asset to identify genes involved in coping with *in vitro* stresses. Studies on pulse ventilation during the *in vitro* period, to balance gas exchange, could be a useful way to reduce this kind of stress and lead to increase in the growth of plantlets *in vitro*. In addition, study on ROS formation during *in vitro* periods and during acclimatization and after acclimatization at the molecular and biochemical level, to detect changes in enzyme activities and gene expression could be very revealing. ROS might be removed by adding selected antioxidants.

All these observations are of use and instrumental to improve tissue culture in plants. The different steps in micropropagation of both woody and bulbous crops are depicted in Table 1. Adopting the results described in this thesis in the different steps during this process will have a positive impact on the speed by which cultures can be established.

Table 1. Different steps in micropropagation of herbaceous and woody plants (left) and of bulbous crops like lily (right) (adapted from De Klerk, 2012).

Herbaceous and woody plants	**Bulbous crops**
Stage 0: preparation of mother plants	Stage 0: preparation of mother plants
Stage 1: initiation	Stage 1: initiation
Stage 2: multiplication	Stage 2: multiplication
Stage 3a: elongation	Stage 3a: bulbing / bulb growth
Stage 3b: rooting	Stage 3b: dormancy breaking
Stage 4: planting and acclimatization	Stage 4: planting

These quantitative improvements of bulblet growth are listed in Table 2. The improvement of growth is shown between brackets as a percentage of the control. It should be noted that not only treatments that directly influence bulblet growth are essential, but also the treatment during the initiation, *viz.*, a stringent control of

contamination. The latter obviously results in a decreased level of microorganisms in the tissues so in more "healthy" cultures.

Table 2. Table summarizing the approaches developed in this thesis to improve lily bulblet growth *in vitro.* The growth increase compared to the non-treated control is in brackets.

- **During sterilization**[1]
  - Reduction of cross contamination (22%)
  - Reduction of hydrostatic pressure related contamination (17%)
- **At the transfer to tissue culture**
  - Selection origin of scale explant
    - Basal and middle scale explants (40-50%)
  - Size of scale explant
    - Large explant (25%)
- **During bulblet growth**
  - Original scale left attached (30%)
  - Mild stress (20-65%)

[1]Increase in addition to the positive effect of a reduced contamination rate

Finally the presented results in this thesis are a further stepping stone towards improving the knowledge about the various steps in tissue culture as well as a way forward to increase the speed by which new cultivars of sufficient high quality can become available to the market.

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Summary

Many geophytes have a high ornamental value. They are preferably propagated by micropropagation because in this way large quantities of uniform, disease-free starting material are produced in a short period of time. In comparison with shoots, bulblets have several clear advantages as starting material. Therefore, *in vitro* bulblet formation is an important target for improvement of tissue culture of geophytes. The research described in this thesis was carried out with the lily cultivars 'Santander' and 'Stargazer'.

Commercially, lily is the second geophyte in the global flower industry. The size of lily bulblets regenerated *in vitro* has a direct effect on the performance in the field. After planting, large bulblets sprout with a stem and gain twice as much weight compared with small bulblets that sprout with a rosette. In the present study we studied basic and applied aspects of the following topics: (1) new methods for sterilization during initiation, (2) the effect of scale-related factors on bulblet growth, (3) growth enhancement by moderate abiotic stresses, and (4) the effect of CO_2 removal from headspace of tissue culture containers on lily bulblet growth and as a control *Arabidopsis thaliana* seedling growth.

Contamination is an everlasting problem in tissue culture laboratories. Lily bulbs are underground organs and contain therefore more contaminants as compared with aerial organs. During initiation, operators cause additional contamination in two ways that have as yet not been recognized adequately. (1) Rinsing explants with sterile water after surface-sterilization is the generally advised method to remove the residues of decontaminants. However, when scales are heavily contaminated, the surface-sterilization does not kill microorganisms in all scales. Surface-sterilization is usually done with batches of 10 to 30 scales and the contaminated scales may cross-contaminate uninfected scales during rinsing in water. We have tested the rinsing water and found heavy bacterial contamination in the 2^{th} and especially the 3^{rd} rinse. The contaminated rinsing water resulted in a high incidence of cross-contamination. Cross contamination was reduced almost fully by rinsing in diluted NaClO solution (0.03%) instead of sterile water. There was no negative effect of diluted NaClO on growth. (2) A second way of introducing contamination by the operator is the entering of microorganisms during detachment from mother bulbs via the vascular bundles caused

by negative hydrostatic pressure within the bulb tissue. By detaching scales from bulbs submerged in 0.03% NaClO, hydrostatic-pressure related contamination was strongly reduced. The growth of bulblets increased by 22% and 17% when cross contamination and negative-hydrostatic pressure related contamination were prevented.

Storage organ formation is controlled by interacting environmental, biochemical and genetic factors. We studied various aspects of the effect of the scale explant. We found that large explants produced larger bulblets than small explants. When bulblets were excised and cultured *in vitro*, growth was improved by 33% when a small piece of the original scale explant was left attached to the bulblet. The position in the scale from where the explant was excised affected the growth of the regenerating bulblets. Basal-scale explants improved bulblet growth by 40-50 % compared with apical-scale explants. This might be related to the physiological state of the tissues: there was more starch and there were more vascular bundles present in basal scale explants. Furthermore, excision of an explant from the middle of the scale improved bulblet growth by 40-50 % compared with explants excised from the edge of the scale. In general, the middle scale explants were heavier and contained wider vascular bundles.

Plants in stressful conditions tend to allocate a higher proportion of biomass to below-ground biomass (roots and storage organs) as compared to above ground biomass. We investigated the effect of moderate abiotic stresses on lily bulblets grown *in vitro*. In general, lily bulblets showed an increased growth after moderate stresses. Hot air increased growth by 30%, hot water by 40%. We also examined the effect of drought and anaerobiosis. Drought stress increased growth of bulblets by 40% in the cultivar ‘Stargazer’, but significantly decreased bulblet growth in cv ‘Santander’. Anaerobiosis increased growth in ‘Stargazer’ and ‘Santander’ by 32% and 65%, respectively. We also showed that a moderate stresses treatment protects lily bulblets against future severe abiotic stresses.

In general, the *in vitro* situation is not favorable for plants. Composition of the headspace (high humidity, strongly fluctuating CO_2- and O_2-levels and accumulation of gases like ethylene), low light, and wounding are unfavorable conditions that plants have to deal with. We examined the effect of CO_2 starvation on growth *in vitro* with and without addition of 3% sucrose to the medium. A CO_2-poor headspace reduced the

growth of bulblets, leaves, roots and scale explants strongly, also in the presence of 3% sucrose. CO_2 removal from the headspace decreased growth of *Arabidopsis* seedlings by 50 % on medium with 3% sucrose. It seems unlikely that the growth reduction on medium with 3% sucrose is caused solely by the lack of sucrose production in photosynthesis when CO_2 is removed. Indeed, we found evidence that the low CO_2 resulted in heavy stress that in turn may reduce growth. F_v/F_m in lily and *Arabidopsis* dropped when CO_2 was removed. Occurrence of reactive oxygen radicals (ROS) was examined in *Arabidopsis* seedlings by staining with nitroblue tetrazolium (NBT). ROS was virtually absent in *ex vitro* growing seedlings and very abundant in seedlings grown under CO_2 starvation. Seedlings grown under normal tissue culture conditions showed an intermediate presence of ROS. We hypothesize that low levels of CO_2 may results in ROS in *in vitro* seedlings which reduces growth.

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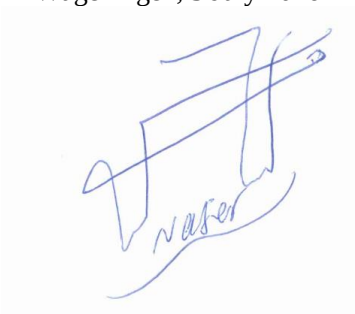
Naser Askari
Wageningen, 5 July 2016



About the author

Naser Askari was born in Rabor, a beautiful mountain city in the south of Iran on March 6, 1977. In 1998, he received an associate degree in plant production from Shahid Bahonar University of Kerman. Subsequently, he finalized in 2000 a BSc in the horticultural crops production department at Tehran University (Aboureyhan Campus). On June 24, 2004 he defended his MSc thesis entitled "Improvement of micropropagation of Gerbera jamesonii" in the horticultural department of Guilan University. Since September 2004, he has been working as lecturer and researcher at Shahid Bahonar University of Kerman.

On March 18, 2010, Naser Askari started a PhD research under supervision of Prof. Dr. Richard G.F Visser and Dr. Geert-Jan De Klerk at the Plant Breeding department of Wageningen University. Naser Askari will continue his research and teaching career as assistant professor in ornamental plants physiology, micropropagation and biotechnology at University of Jiroft in Iran.

Publications

Askari, N., Wang, Y., & de Klerk, G. J. (2014). In tissue culture of lilium explants may become heavily contaminated by the standard initiation procedure. Propagation of ornamental plants, 14(2), 49-56.

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EPS Certificate

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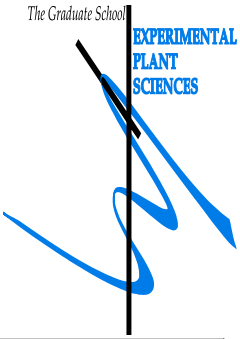

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1) Start-up phase	*date*
► **First presentation of your project** Elucidation and improvement of bulb induction and bulb growth in tissue culture of lily and other bulbous crops	Feb 02, 2012
► **Writing or rewriting a project proposal** Elucidation and improvement of bulb induction and bulbgrowth in tissue culture of lily and other bulbous crops	2010
► **Writing a review or book chapter**	
► **MSc courses**	
Plant Biotechnology (GEN 20806)	Nov-Dec, 2012
Gene Technology (MOBB 20306)	Sep-Nov, 2015
Genomics (ABG 30306)	Nov-Dec, 2015
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

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EPS theme 3 'Metabolism and Adaptation', Utrecht University	Apr 26, 2012
EPS theme 3 'Metabolism and Adaptation', Wageningen University	Mar 12, 2014
► **Lunteren days and other National Platforms**	
Annual Meeting Éxperimental Plant Sciences, Lunteren, NL	Apr 04-05, 2011
Annual Meeting Éxperimental Plant Sciences, Lunteren, NL	Apr 02-03, 2012
Annual Meeting Éxperimental Plant Sciences, Lunteren, NL	Apr 22-23, 2013

►	**Seminars (series), workshops and symposia**	
	European Retreat of PhD Students in Experimental Plant Sciences, Paris, France	Jul 05-08, 2011
	European Retreat of PhD Students in Experimental Plant Sciences, Norwich, UK	Aug 14-17, 2012
	Plant Research day (Plant Breeding)	2010
	Plant Research day (Plant Breeding)	Mar 08, 2011
►	**Seminar plus**	
►	**International symposia and congresses**	
	VII International Symposium on In Vitro Culture and Horticultural Breeding: IVCHB, Ghent, Belgium	Sep 18-22, 2011
	In Vitro Biology Meeting, Providence, Rhode Island, USA	Jun 15-19, 2013
►	**Presentations**	
	Rapid propagation of tulip in tissue culture ,TTIG symposium, NL (Poster)	Sep 21, 2011
	Elucidation and improvment of bulb induction and bulb growth in tissue culture of lily, France (Poster)	July 05-08, 2011
	Effect of GA and cold treatments on bulb growth of lily, Lunteren (Poster)	Apr 02-03, 2012
	Lily bulb growth in tissue culture: the role of the explants, UK (Poster)	Aug 14-17, 2012
	The role of photosynthesis on the growth of lily bulblets in vitro, USA (Poster)	Jun 15-19, 2013
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	Meeting with a member of the International Advisory Board of EPS	Nov 19, 2012
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Increasing photosynthesis in plants	Aug 21-26 2011
Microscopy and spectroscopy in food and plant science	May 07-11 2012
Natural variation in plants	Aug 21-24 2012
► **Journal club**	
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4) Personal development	*date*
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