Floriculture and Ornamental Biotechnology ©2012 Global Science Books

Micropropagation of Bulbous Crops: Technology and Present State

Geert-Jan de Klerk

Wageningen UR Plant Breeding, PO Box 16, 6700 AA Wageningen, The Netherlands

Corresponding author: **geertjan.deklerk@wur.nl**

ABSTRACT

Conventional propagation of bulbous crops must be supplemented with micropropagation to satisfy the requirements of present-day horticulture with respect to fast production of disease-free, superior starting material. Adequate micropropagation protocols for bulbous crops are therefore a *sine qua non*. The successive steps in micropropagation of bulbous crops are reviewed: initiation, multiplication, bulb formation, dormancy breaking and planting. In the first two steps, new shoots or bulblets are generated by axillary bud outgrowth or adventitious regeneration. During initiation, endogenous contamination may be a severe problem since bulbs grow subterraneously and have often been propagated vegetatively in the field for many years. Other drawbacks are insufficient axillary branching, poor adventitious regeneration and inferior growth. The latter, inferior growth, is likely the most significant problem and is caused by poor translocation of medium ingredients to the growing regions within the explant. In micropropagation of bulbous crops, bulblets should be produced because of, among others, easy handling and acclimatization. For optimal performance after planting in soil, preparatory treatments are required in particular a dormancy breaking treatment. A phase-change from juvenile to adult and protective pretreatments are also profitable. It is concluded that when major problems like that of inferior growth have been solved, commercial micropropagation of bulbous crops will experience a second heyday.

Keywords: micropropagation, bulbous crops, tulip, lily, zantedeschia, dahlia, narcissus
Abbreviations: ABA, abscisic acid; **BAP**, 6-benzylaminopurine; **CT**, cold treatment; **GA**, gibberellin; **MS**, Murashige-Skoog medium; **NAA**, 1-naphthaleneacetic acid; **WWT**, warm-water treatment

CONTENTS

INTRODUCTION

In present-day horticulture, starting material has to satisfy stringent requirements: Propagules must be vigorous, homogeneous and free from diseases. These requirements are difficult to meet in bulbous crops using only conventional vegetative propagation in the field because of phytopathological and physiological deterioration. Phytopathological deterioration is caused by the persistence of pathogenic micro-organisms during conventional vegetative propagation: Once a pathogen has entered a plant, it usually persists during the following vegetative generations. Conventionally produced starting material is therefore often populated endogenously by micro-organisms. This holds especially for crops with subterraneous storage organs in which the storage organs are used for vegetative propagation. In sexual propagation, on the contrary, the seed acts as a barrier that inhibits the spread of most micro-organisms in time (from one generation to the next). When propagation is carried out *in vitro*, disease-free propagules are produced

Received: 16 February, 2011. Accepted: 20 December, 2012.

Invited Review

provided the proper measures have been taken. Physiological deterioration is caused by epigenetic changes (Smulders and De Klerk 2011). Sexual propagation resets the epigenetic status and invigorates the plants. The same can be achieved by propagation *in vitro* especially when a step of adventitious regeneration is included (Smulders and De Klerk 2011).

Micropropagation is also a *sine qua non* for breeding. The increasing demands of society and consumers with respect to disease resistance, new colours and new architecture require a rapid introduction of newly produced cultivars onto the market. Conventional vegetative propagation of bulbous crops is slow. In tulip, it takes 20-30 years to produce a commercial batch of a newly developed cultivar. Micropropagation speeds up vegetative propagation considerably. Introduction of a newly bred lily cultivar on the market used to take up to 15 years but because of the high propagation rates *in vitro*, newly bred cultivars can now be introduced on the market within 7-8 years (Langens-Gerrits 2003).

For all major bulbous crops, micropropagation protocols have been developed (Kim and De Hertogh 1997), but many are not enough workable in commercial practice. Main drawbacks are, just like in other crops, the high price of tissue-cultured propagules (because of the high input of labour), poor propagation of many genotypes, and the occurrence of off-types. In bulbous crops, tissue culture is applied commercially in particular in lily and zantedeschia. Other bulbous crops are micropropagated in much smaller numbers or not at all. In this review, the successive steps in micropropagation of bulbous crops are discussed. It seems to be generally believed that on the whole micropropagation of bulbous crops has been finished off. Thus, research would only concern minor adjustments in previously developed protocols. In the final sections of this review, major general barriers will be discussed. Removal of these obstacles will lead to novel micropropagation procedures of bulbous crops and when these major drawbacks have been solved, it is a matter of time until micropropagation is used at a large scale for all major bulbous crops. At the same time, when the problems are not solved, several major bulbous crops may become marginalized.

In this review, the term "bulbous crop" is used in a broad sense. From the botanical perspective, bulbs are defined as underground organs typified by scales (scales are swollen leaves or petioles), so the term "bulbous crops" should be restricted to crops with such organs. However, all ornamental geophytes (a geophyte is defined as a perennial plant with an underground storage organ) are commonly called "flower bulbs" or "bulbous crops" so crops with corms, rhizomes, tubers, or tuberous roots, as crocus, dahlia, zantedeschia or cyclamen, are also considered to be bulbous plants (De Hertogh and Le Nard 1993).

THE STEPS IN MICROPROPAGATION OF BULBOUS CROPS

In micropropagation of herbaceous and woody crops, five steps are distinguished, namely, motherplant preparation, initiation, multiplication, rooting and acclimatization (George and Debergh 2008). During the multiplication step, shoots (microcuttings) are produced. They are rooted *in vitro* or *ex vitro* and require acclimatization after planting in soil. In micropropagation of bulbous crops, preferably bulblets are produced rather than shoots. Because of this, the successive steps in micropropagation of bulbous crops are somewhat different (**Table 1**). In bulbous crops, bulblets may be produced 'automatically' during the multiplication step. When shoots are produced, they are treated to produce bulblets. Often, bulblets do not require a rooting treatment and acclimatization, but they do require a dormancy-breaking treatment before planting. Below the successive steps are discussed. **Table 2** specifies the common procedures of initiation and multiplication for various major bulbous crops.

Table 1 Major steps in micropropagation of herbaceous and woody plants (according to George and Debergh 2008) and the corresponding steps in bulbous plants.

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

Step 1: Initiation

1. Axillary, adventitious, and semi-axillary propagation

Plants contain one or more apical (terminal) buds (buds at the end of a main shoot or side shoots), and many axillary buds. For conventional vegetative propagation, side shoots (shoots originating from the outgrowth of axillary buds) are excised and rooted. In lily, for example, axillary buds on stems may grow into bulbils that are excised and used as propagules. The basal plate of a bulb is a very abbreviated shoot. In tulip grown in the field, bulbs generate daughter bulbs by outgrowth of axillary buds from the basal plate. Apical and axillary buds may be used for initiation in tissue culture (**Fig. 1A, 1B**). In tissue culture of, for example, zantedeschia, axillary buds are excised from tubers (Cohen 1981). In true bulbous crops, apical and axillary buds have been used occasionally, for example, in tulip (Ghaffoor *et al*. 2004) and iris (M. Van Schadewijk pers. comm.). The advantage is that shoots derived from these buds are large.

Alternatively, tissues without pre-existing buds may be used and new apical buds are induced adventitiously. Well-known examples in conventional horticulture are propagation of lily from excised scales ("scaling") and hyacinth by scooping.

In tissue culture (**Fig. 1C, 1D**), adventitious buds may be induced from many types of tissue but in most micropropagation protocols adventitious buds are induced from scales (for example in lily, **Fig. 2**) or flower stems (for example in tulip). Taking explants from flower stems has various advantages among others relatively low endogenous contamination (Ziv and Lilien-Kipnis 2000). In addition, often twin scales (two adjacent scales connected by a portion of the basal plate) are used and shoots/bulblets are generated from in between (**Fig. 3**). Between scales axillary buds occur (only a few in each bulb) and most newly generated shoots/bulblets do not originate from these axillary buds but from distinct groups of cells at the abaxial side of scales near the basal plate (allium, Kahane *et al*. 1992). At the abaxial side of scales of nerine near the basal plate, these cell groups are meristematic (Grootaarts *et al.* 1981). Because they originate in the axil-area from anatomical distinct groups of cells that have a meristematic appearance,

Table 2 Pathways for micropropagation of some bulbous crops. It should be noted, that most can also be micropropagated in other ways. Nerine, for example, may also be initiated from flower stems.

Starting material	Explant during multiplication	Type of shoot formation	Examples
fragment cut from scale	fragment cut from scale	adventitious	lily, hyacinth
slice cut flower stem	slice cut from shoot	adventitious	tulip
twin scales	shoot clusters	axillary and semi-axillary	narcissus, iris, nerine
axillary buds ('eyes') cut from tubers	shoots	axillary	zantedeschia

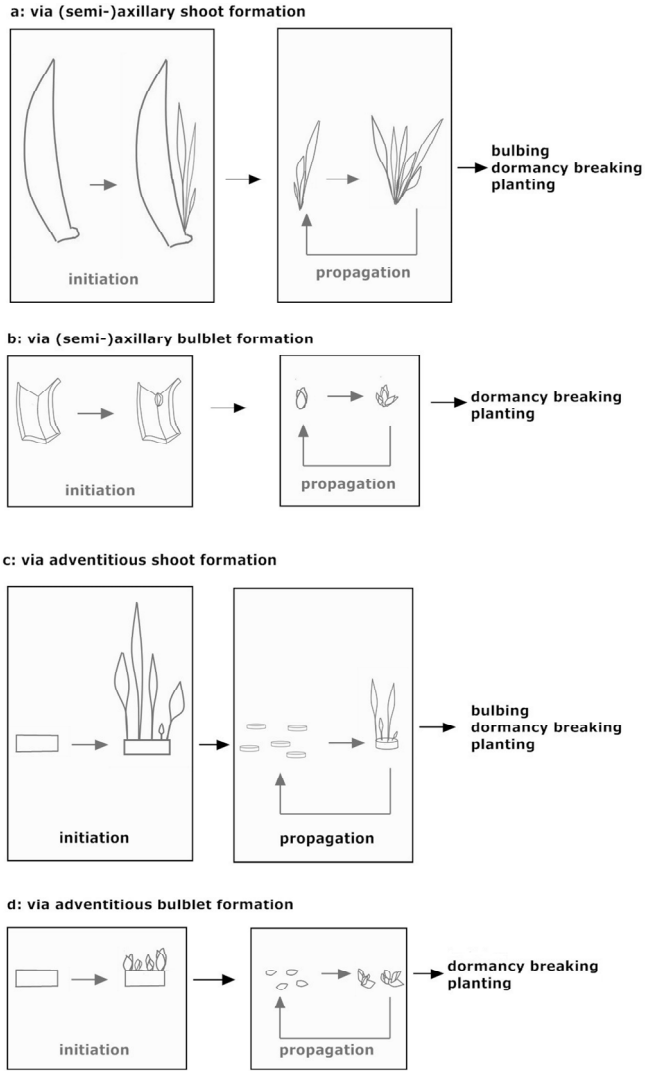

Fig. 1 Micropropagation schemes for bulbous crops.

Fig. 2 Adventitious bulblet formation from lily scale explants. Left 4 weeks and right 11 weeks after the start of culture of scale fragments on nutrient medium (MS, 3% sucrose, 0.54 µM NAA). Note that the adventitious bulblets are formed at the basal edge of the explant. This is caused by basipetal auxin transport (Van Aartrijk and Blom-Barnhoorn 1984).

this type of propagation may be denoted as 'semi-axillary'. However, it is usually classified as adventitious regeneration. **Fig. 4** shows the position of such meristematic cells in nerine (adapted from Grootaarts *et al.* 1981). On base of hormonal requirements, it was concluded for *Urginea maritima* that outgrowth of the semi-axillary buds mainly resembles adventitious regeneration (Shahin *et al.* 2013). It should be noted that in monocotyledons, axillary buds are generated at a similar (abaxial) position (Sharman 1945; Evert 2006).

Both adventitious and axillary propagation are used in conventional vegetative propagation. As a matter of fact,

Fig. 3 Narcissus twin scales. Left 4 weeks and right 12 weeks after the start of culture of scale fragments on nutrient medium (MS, 3% sucrose, 0.54 µM NAA, 4.4 µM BAP).

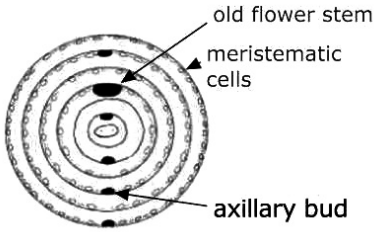

Fig. 4 Semi-axillary propagation in nerine. A schematic drawing is shown from a cross-section of a bulb after 21 days of culture in which the black elliptical spots represent pre-existing meristems (axillary buds) and the biggest black elliptical spot an old flower stem. The small open spots represent groups of meristematic cells. (Adapted from Grootaarts *et al.* 1981).

methods for initiation of the various bulbous crops *in vitro* have often been "borrowed" from conventional vegetative propagation. Both in axillary and adventitious propagation, the new shoots may be genuine shoots or bulblets (= shoots consisting of swollen leaves). This depends largely on the genotype but can be modified by the tissue-culture conditions. In lily, bulblets are formed under normal conditions, but when fluridon (an inhibitor of ABA synthesis) is added during regeneration, scale formation is completely inhibited and a rosette plantlet is generated composed of leaves with nonswollen petioles attached to the basal plate (Kim *et al.* 1994). From flower stem slices of tulip, shoots are regenerated.

2. Contamination

The surface of plants is always populated by micro-organisms. They can be efficiently removed by surface-sterilization, for example, by a treatment with a diluted hypochlorite solution. Contaminating micro-organisms also occur within plant tissues in the vascular system, between cells and even within cells. These micro-organisms often reduce plant growth. Many bacteria species do not grow well on tissue culture media and stay hidden inside the plant tissue during the culture *in vitro* (Leifert and Waites 1992). With the lapse of time during many subculture cycles, some may adapt to tissue culture media and after all colonize the medium. In bulbous crops, especially when starting with explants excised from bulbs, endogenous contamination is often a major, insuperable problem. Antibiotics are widely used. However, they do not kill micro-organisms living within the tissue but only impede their growth in the medium. This is because the antibiotics do not attain a sufficiently high concentration in the target tissues (just as with nutrients, translocation of antibiotics to the target regions is problematic; see the discussion on nutrient translocation later).

Endogenous contamination may be overcome by a warm-water treatment (WWT) (Hol and van der Linde 1992; Langens-Gerrits *et al.* 1998). The temperature varies

depending on the species from 43°C to over 50°C and the duration of the treatment from 1 to 4 h. It is believed that at the high temperature, the contaminating organisms die whereas the plant tissues survive (Grondeau and Samson 1994). A WWT can only be given to tissues that have sufficient resistance to heat stress, so to bulbs but also to dormant buds of woody plants (Langens-Gerrits *et al.* 1998). Nevertheless, a WWT of bulbs still involves high stress and damages the plant tissues. Because of this damage, the explants may produce toxic gases and may not survive when the container has been tightly closed (Langens-Gerrits *et al.* 1998).

Step 2: Multiplication

1. Axillary, adventitious and semi-axillary multiplication

The multiplication phase contributes the most to the multiplication rate *in vitro*: Each year, various cycles can be completed and by addition of hormones the multiplication rate per cycle is high. In the field, the propagation cycle is linked to the seasons so each year only one cycle can be performed. Major problems in bulbous crops are slow growth and lessening of growth with successive cycles. In daffodil, which is propagated by semi-axillary branching, the decrease of growth was overcome by intermittent cycles of bulb formation and mini-twinscaling (Langens-Gerrits and Nashimoto 1997).

For the formation of new shoots/bulblets during step 2, the same pathways are used as in initiation (**Fig. 1**), so adventitious regeneration, axillary multiplication and semi-axillary multiplication. The propagules during step 2 may be a bulblet or a shoot. In lily, for example, bulblets are produced from scales of microbulblets. In tulip, shoots are regenerated from slices cut from shoots. It is believed that multiplication via shoots is faster (Van Aartrijk and Van Der Linde 1986).

2. Liquid medium

There are various advantages of liquid medium as compared to semisolid agar-media. These advantages include (1) that agar is expensive and contains impurities, (2) that in liquid medium, components not only move by diffusion but also by convection (by means of turbulences), (3) that liquid medium reduces labour costs among others by easier transfer (when subculturing, the plantlets do not have to be positioned), and (4) that in liquid medium a large surface area of the explant is involved in uptake and that the distances of translocation within the plantlets are strongly reduced. With respect to the latter, it should again be noted that translocation of medium ingredients within the explant is problematic (De Klerk 2010, and see below). Uptake through the epidermis may occur via the stomata and via aqueous pores (Schönherr 2006). It should also be noted that most advantages are expected with shoot cultures as bulbs have a small surface-to-volume ratio and since the surface of scales is likely less permeable. Indeed, in hippeastrum, liquid medium has hardly any stimulating effect on bulblet growth (Ilczuk *et al*. 2005).

The major problem associated with liquid medium is poor gas exchange when tissues are submerged (Jackson 1985). Another disadvantage of liquid medium is the increased incidence of contamination. The problem of suffocation may be solved by medium agitation but then plant tissues may be damaged by shear. Nerine and gladiolus have been propagated in liquid medium by means of meristematic clusters (Lilien-Kipnis *et al.* 1994; Ziv 1990; Ziv *et al.* 1994). Shear damage was reduced by adding GA-synthesis inhibitors so that only meristematic, nodule-like structures occurred and leaf formation was impeded. This system has been used for many crops. Before planting, sometimes GA has to be added to overcome the after-effects of the GA-synthesis inhibitors. Other methods to solve the suffocation problem include temporary immersion (Etienne and Berthouly 2002) or use of a shallow layer of liquid medium so not all tissue is submerged. **Fig. 5** shows growth of dahlia cuttings on semi-solid medium and in a shallow layer, nonagitated liquid medium. Growth in liquid medium is much faster with respect to the increase of dry and fresh weight, and the propagation factor.

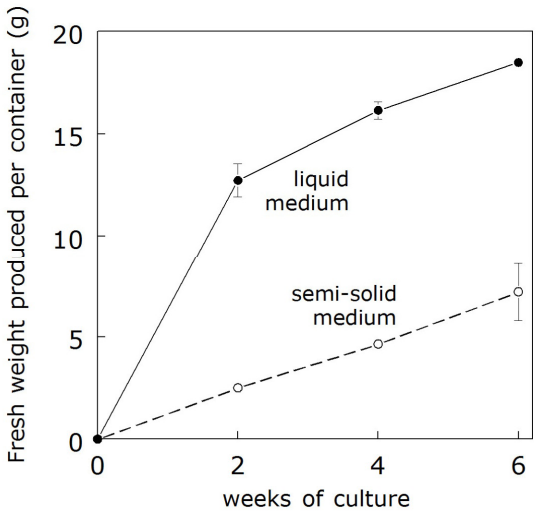

Fig. 5 Growth of dahlia on semisolid medium (0.7% agar) and in liquid medium. (Adapted from De Klerk and Ter Brugge 2011).

Multiplication using singulated somatic embryos induced from cell suspensions in liquid medium is the preferable method of micropropagation also in bulbous crops, but it should be noted that this may lead to epigenetic or genetic variation. Somatic embryogenesis in bulbous crops has been reported (tulip: Gude and Dijkema 1997; Koster 1993; freesia: Bach 1992; nerine: Lilien-Kipnis *et al*. 1994), but unfortunately not in liquid systems with the notable exception of embryogenic suspension cultures of daylily (Krikorian *et al*. 1986).

Step 3a: Bulbing

When in bulbous crops shoots are generated, they may be rooted and planted in soil. These shoots form bulblets during the first growing season. However, in iris a significant percentage planted shoots is incapable of bulblet formation (Van Der Linde and Schipper 1992) and the same may hold for other bulbous crops. Other advantages of bulblets are that they are more robust than shoots and easier to handle, and that they do not require acclimatization. Bulblets may be formed 'automatically' without an additional treatment (for example in lily) or generated shoots are given an additional bulb-inducing treatment, usually, a cold treatment (Van Aartrijk and Van Der Linde 1986). In the latter case, bulblet formation *in vitro* requires much labour and takes a long time (usually several months). In this volume, bulb formation will be dealt with in another chapter (Podwyszyńska 2012).

Step 3b: Dormancy breaking

When bulblets are produced, they are usually dormant, *i.e*., they are unable to sprout even though the conditions (temperature and water availability) are suitable. They first need a dormancy-breaking treatment to enable sprouting. In lily, dormancy does not develop when fluridone, an inhibitor of ABA synthesis, has been applied during regeneration (Kim *et al.* 1994) demonstrating that ABA is required for dormancy development. ABA is also required for bulb formation (see before). It is interesting to note that in *Arabidopsis* seeds that are incapable of ABA synthesis by mutation dormancy also does not develop (Karssen *et al.* 1983). The types of dormancy in *Arabidopsis* seeds and lily bulblets are very different: In *Arabidopsis* seeds, dormancy is broken by

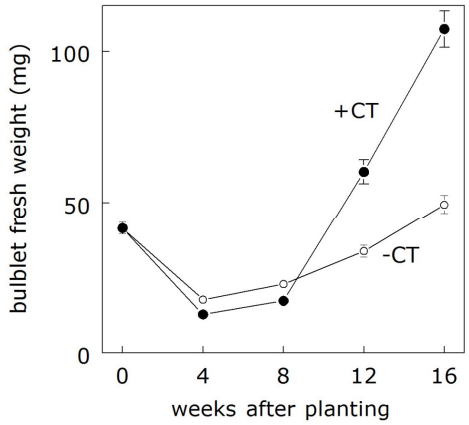

Fig. 6 Growth of lily bulblets after planting in soil. The bulblets had been generated at 15°C and had (+ CT) or had not (- CT) been given a cold treatment of 6w at 5°C. Both the cold-treated and noncold-treated bulblets sprouted to almost 100% (so both are considered as nondormant) but growth in the cold-treated ones was much faster. (Adapted from De Klerk et al., 1992).

storage of dry seeds for a few months at room temperature (afterripening). In lily bulblets, dormancy is broken by storage of bulblets at low temperature (a few weeks at 5°C; similar to stratification of seeds).

A cold treatment to break dormancy is used in many bulbous crops. In iris, though, dormancy is broken by storage (several weeks) at high (30°C) temperature (Van Der Linde and Schipper 1992). In daffodil and nerine, a rooting treatment *in vitro* is required to achieve high sprouting after planting (Langens-Gerrits and Nashimoto 1997; Paffen and Langens 2003).

After careful experimentation several conventional ideas about dormancy in seeds appeared to be incorrect and the same holds for bulbous crops:

- The general definition of dormancy as a general arrest of growth (see Kamerbeek *et al.* 1970 for bulbs) is mistaken. Dormant seeds have a high metabolic activity. After the development of dormancy, lily bulblets continue to generate new scales at the same rate as before (Delvallée *et al.* 1990). In lily, the induction of dormancy does not correspond to a decrease of metabolism but to a switch in the development of the primordium: the primordium can no longer develop into a leaf, but only into a scale.
- Dormancy is often typified as an all-or-nothing phenomenon. This is not correct. Already in 1940, Borriss, working with seeds of *Agrostemma githago*, noted that dormant seeds do germinate but only at a restricted range of conditions (Borriss 1940). Dormancy breaking treatments widen this range. Thus, for Borriss, dormancy was not absolute but relative (relative to the environmental conditions at which the seeds are imbibed.) The same was found for lily bulblets: Lily bulblets are dormant at certain environmental conditions but the same bulblets sprout at other conditions (De Klerk and Paffen 1995).
- The dormancy-breaking treatment not only promotes sprouting. Lily bulblets generated at 15°C are able to sprout at standard conditions to almost 100% without a preceding cold treatment but their growth after planting is much improved by a cold treatment (**Fig. 6**). Treatments like soaking bulblets overnight in a solution of GA breaks dormancy as shown by near 100% sprouting but at the same time results in poor growth after planting compared with bulblets in which dormancy had been broken by a cold treatment. This is caused by hydrolysis of macromolecular reserves during the cold treatment to usable small molecules that can be easily consumed by the growing leaf (Langens-Gerrits *et al.* 2003b).

Fig. 7 Lily bulblets 10 weeks after planting in soil. The bulblet left has sprouted as a rosette in the juvenile mode and the bulblet right has sprouted with a stem in the adult mode.

Step 4: Planting in soil

Researchers developing micropropagation protocols only occasionally scrutinize transfer to *ex-vitro* conditions and usually ignore the effects of tissue culture conditions on subsequent acclimatization. However, during tissue culture propagules can be prepared for fast growth after transfer to soil; in other words, during tissue culture a developmental or physiological state can be generated that promotes growth after transfer *ex vitro*. As noted in the previous section, this firstly refers to dormancy breakage. Other possibilities concern the induction of phase change and stress resistance.

1. Phase change

Lily bulblets produced *in vitro* may sprout as a rosette with one or more leaves. These bulblets are juvenile (Langens-Gerrits *et al.* 2003a). Bulblets may also sprout in an adult mode with a stem with elongated internodes and many leaves (**Fig. 7**). Sprouting in the adult mode is advantageous, as bulb growth after planting is more than twice as fast (Langens-Gerrits *et al.* 2003a). Large bulblets sprout more often with a stem than small bulblets. Thus, during tissue culture large bulblets have more often made the transition from juvenile to adult (the phase change) in comparison with small bulblets. When bulblets from the same size are taken (for example the size-class 200-300 mg), some tissue culture conditions promote phase change irrespective of their effect on weight. Bulblets regenerated from large explants on medium with a high sucrose concentration or on medium with a low salt concentration more often make the phase change (Langens-Gerrits *et al.* 2003a). Such 'programming' of bulblets during tissue culture may be very helpful for tissue culture of bulbous crops and requires more research.

2. Stress resistance: protective compounds

When plants are under stress, they produce protective compounds, including proline, betaine, putrescine and trehalose. When applied exogenously, these compounds may also protect plant tissues from stress. Thus, when they are added to Arabidopsis seedlings before abiotic stress, survival increases often from close to 0% to 80-100% (De Klerk and

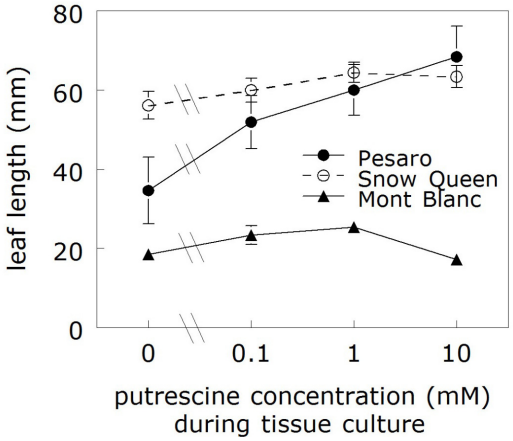

Fig. 8 Effect of culture with addition of putrescine, a protective compound, in three lily cultivars. Bulblets were regenerated from scale explants under standard conditions with increasing concentrations of putrescine. After 12 weeks, they were harvested, cold-treated at 5°C for 6 weeks and planted. At planting there were no weight differences between the various concentration of prutrescine. After 6 weeks in soil, the length of the longest leaf was measured (Unpublished results of A. Ptak and G.J. de Klerk).

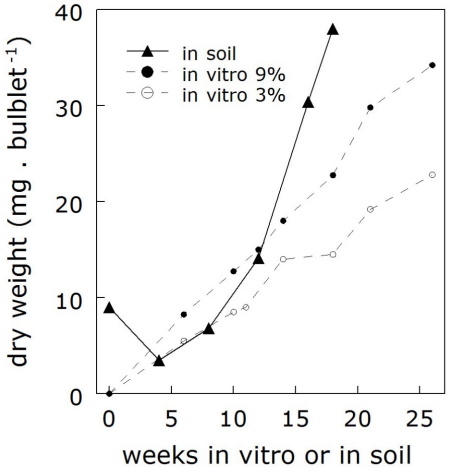

Fig. 9 Dry weight of lily bulblets after increasing periods of culture in soil or *in vitro*. The bulblets cultured in soil had been regenerated *in vitro* for 11 weeks at 20°C and then, before planting, been cold-treated. They were cultured at 17°C in a culture room with 16 h light per day (40 $\mu E.m^{-2}.sec^{-1}$). The bulblets regenerating from scale explants were at 20°C and 3% or 9% sucrose. During the period of fastest growth (12 to 18 weeks), growth of bulblets in soil was *ca*. 3 times faster than growth *in vitro* at 9% sucrose.

Pumisutapon 2008). When protectants are added to rose cultures during the *in vitro* rooting stage, they show increased survival and growth after transfer to soil (De Klerk 2002). Bulblets are more robust than shoots. Nevertheless when the protectant putrescine was applied to lily cultures, bulblets displayed increased performance (**Fig. 8**).

MAJOR OBSTACLES: BACKGROUNDS AND PROSPECTS

The low rate of multiplication is a major setback in micropropagation of bulbous crops. The major causes are: (1) poor generation of new buds due to inferior adventitious regeneration or little axillary branching and (2) a low relative growth rate. Other main problems include persistent (endogenous) contamination, occurrence of off-types and - as in all other crops- the high prices of micropropagules. In the following sections, slow multiplication and off-types are briefly discussed.

Poor formation of new buds

Depending on the pathway (**Fig. 1**), both during the initiation and multiplication phase, new apical buds are generated adventitiously or by outgrowth of axillary buds. The rate of formation of adventitious buds depends on the ability of the starting tissue to regenerate and the hormonal additions in tissue culture. With respect to the second factor, most research concerns the level and type of cytokinins and auxins. As the understanding of the process of adventitious regeneration is still limited, researchers rely on the strategy of 'spray and pray'. However, more and more basic research is being carried out which eventually may lead to advanced regeneration protocols. A promising recent insight is that the regenerative process is composed of distinct steps each with specific hormonal requirements (Christianson and Warnick 1983; De Klerk 2009; Sena and Birnbaum 2010). With respect to the starting tissue, there are rules of thumb regarding the physiological age (young tissues perform better), the ontogenic age (juvenile tissues perform better), and the dormancy status (nondormant tissues perform better). Scientific research on the underlying mechanisms is scarce or lacking.

Poor axillary branching is caused by strong apical dominance. Apical dominance is the control exerted by the shoot apex over the axillary buds. The generally accepted underlying mechanism involves auxin (that is transported downwards from the shoot apex and inhibits axillary bud outgrowth indirectly) and cytokinin (that is transported upwards from the roots and acts as stimulator). This mechanism has recently been adjusted (review in Ongaro and Leyser 2008). Analysis of branching mutants in *Arabidopsis*, pea and petunia has presented evidence for a novel branching-inhibiting, carotenoid-derived hormone. Recently, this novel hormone has been identified in pea (Gomez-Roldan *et al.* 2008) and in rice (Umehara *et al.* 2008) as strigolactone. By adding the carotenoid-synthesis inhibitor fluridone, a significant enhancement of axillary branching in tissue culture has been achieved in apple (De Klerk 1992) and in alstroemeria (P. Pumisutapon and G.J. de Klerk unp. results).

Inferior growth

Because of the favourable conditions in tissue culture (ample water, abundant organic and inorganic nutrients, adequate temperature) one would expect maximal growth *in vitro*. Nonetheless, growth is at best similar to growth in the field (De Klerk 2010). **Fig. 9** shows as an example growth of bulblets in tissue culture compared with growth *ex vitro* in a climate room with inadequate light conditions (three 33W fluorescent tubes). Why is growth in tissue culture less than expected? This is most probably related to poor translocation of medium ingredients to the growing areas in explants. In the following paragraphs this will be justified using theoretical grounds.

First it should be noted that solutes (compounds dissolved in water) are translocated in two ways: (1) by diffusion and (2) by hitching with the water flow. Diffusion is driven by random thermal agitation and is fast over short distance, but very slow over large distance. According to Fick's law of diffusion, diffusion over 50 µm takes 2.5 sec, over 2 cm one week, and over 1 meter 32 years! Another factor that contributes to low translocation by means of diffusion is the small diameter of the tissue through which diffusion occurs. For these reasons, plants use water flow in the vascular tissues for long distance transport (Taiz and Zeiger 2006).

In tissue culture, long-distance transport (from the medium to the growing regions of the shoot, usually a few cm) depends also upon water flow in the vascular bundles. Under normal conditions, the movement of water in the xylem is driven by transpiration and root pressure. In tissue culture containers, the relative humidity is almost 100% (99.5% close to the stomata; Chen 2004), so transpiration is

expected to be little. Transpiration from leaves of shoots cultured *in vitro* has only been measured seldom and is a few per cent of the transpiration rate in the field (Tanaka *et al*. 1992; De Klerk 2010). This may be insufficient to support adequate nutrient supply. Moreover, soon after the explants have been cut wound tissue is formed and likely acts as a barrier to reach the xylem flow.

Under natural conditions, the phloem supplies nutrients to the growing tissues. Water flow in the phloem is brought about by differences in osmotic potential at the source and the sink caused by loading and unloading of sucrose, respectively. Phloem functions differ according to 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). About the functioning of phloem in tissue culture nothing is known. 'Normal' phloem transport in tissue culture seems unlikely since photosynthesis (which is the driving factor at the source) is low in tissue culture.

In conclusion, on theoretical grounds both xylem and phloem probably malfunction in tissue culture and seem therefore to be unable to supply the growing tissues with sufficient nutrition. The validity of the argumentation in the preceding paragraphs is shown by the sharply increased growth in liquid medium, namely in temporary immersion bioreactors (Etienne and Berthouly 2002) and in static liquid medium (De Klerk and Ter Brugge 2011, and **Fig. 5**). In liquid medium, translocation of ingredients from the medium to the regions of utilization is facilitated by the short distance from the site of uptake (leaves) to the areas of growth. Medium ingredients are taken up by leaves via stomata and aqueous pores. Here it should be noted that the epidermis is very impermeable compared to the cut surface. Since in bulblets the surface-volume ratio is small, liquid medium unlikely stimulates bulblet growth unless the bulblets have roots or leaves.

Off-types

Aberrant micropropagated plants have been frequently observed. There are various causes: (1) genetic changes (changes in the DNA sequence; this is also referred to as somaclonal variation); (2) epigenetic changes (long-lasting changes in the expression of the information in the genome); (3) chimeral segregation and (4) spontaneous loss of pathogens in particular of viruses (Smulders and de Klerk 2011).

When plants are produced by means of adventitious regeneration, especially via an intermediate callus phase, often an increased incidence of somaclonal (genetic) variation is observed (De Klerk 1990). In commercial lily micropropagation, leaves with white stripes have been noticed (cultivar White Mountain). After a protracted intermediate phase of callus growth, a notable percentage of off-types has been reported (Van Harmelen *et al.* 1997). Generally, though, in lily variation has been recorded only very rarely, even though lily is propagated adventitiously.

Epigenetic variation is observed after both adventitious and axillary propagation. Zantedeschia is propagated via axillary branching. In some cultivars, occurrence of bushy plants is a major problem (D'Arth *et al*. 2002). This variation is epigenetic: It is not caused by a change in the genetic information but by altered expression of the genetic information, probably related to DNA-methylation and/or histone modifications (Smulders and de Klerk 2011). Epigenetic changes have been observed frequently in tissue culture, for example, tissue proliferation in rhododendron (Brand *et al.* 2000), bushiness in gerbera (Topoonyanont and Debergh 2001), and flower malformation in oilpalm (Eeuwens *et al*. 2002). Tissue culture practices underlying epigenetic changes include high cytokinin levels in the medium and short subculture cycles during the multiplication step.

CONCLUDING REMARKS

Conventional vegetative propagation supplemented with micropropagation is the only way to provide the bulb growers with starting material that meets the requirements of present-day horticulture. The micropropagation industry adequately uses the results of tissue-culture research carried out during the first 5 or 6 decades of the previous century. This research concerned asepsis, organic and inorganic nutrition, plant growth regulators, and the use of support systems. On this base, the micropropagation industry has expanded from 130 million plantlets produced worldwide in 1986 to some 1-1.5 billion produced today (Prakash 2009). Research, though, has not progressed rapidly anymore. Many of the recent scientific developments in plant tissue culture concern only protocols for 'new' genotypes, but research lacks significant breakthroughs on general bottlenecks discussed in the previous section. Some basic experimentation has been initiated on adventitious regeneration and apical dominance (see before) and the results require translation to micropropagation. To solve the problem of the low growth rate, research is needed on the barriers to growth that occur in tissue culture (De Klerk 2010). Many users and researchers feel that the micropropagation technology has finished off and that about all has been reached that is possible. However, applications of new basic research will undoubtedly result in a second boost to micropropagation.

ACKNOWLEDGEMENTS

This work was partly financed by the Dutch Product Board for Horticulture (Productschap Tuinbouw).

REFERENCES

Bach A (1992) Somatic embryogenesis from zygotic embryos and meristems of *Freesia hybrida*. *Acta Horticulturae* **325**, 429-434

Borriss H (1940) Über die innere Vorgänge bei der Samenkeimung und ihre Beeinflussung durch Aussenfaktoren. *Jahrbuch Wissenschaftliche Botanik* **89**, 254-339

Brand MH, Ruan Y, Kiyomoto R (2000) Response of *Rhododendron* 'Montego' with 'tissue proliferation' to cytokinin and auxin *in vitro*. *HortScience* **35**, 136-140

Chen CC (2004) Humidity in plant tissue culture vessels. *Biosystems Engineering* **88**, 231-241

Christianson ML, Warnick DA (1983) Competence and determination in the process of *in vitro* shoot organogenesis. *Developmental Biology* **95**, 288-293

Cohen D (1981) Micropropagation of *Zantedeschia* hybrids. *International Propagator's Society Combined Proceedings* **31**, 312-316

D'Arth SM, Simpson SI, Seelye JF, Jameson PE (2002) Bushiness and cytokinin sensitivity in micropropagated *Zantedeschia*. *Plant Cell, Tissue and Organ Culture* **70**, 113-118

De Hertogh AA, Le Nard M (Eds) (1993) Botanical aspects of flower bulbs. In: *The Physiology of Flower Bulbs*, Elsevier, Amsterdam, pp 7-20

De Klerk GJ (1990) How to measure somaclonal variation: A review. *Acta Botanica Neerlandica* **39**, 129-144

De Klerk GJ (1992) Hormonal control of dormancy and apical dominance in tissue-cultured plants. *Acta Botanica Neerlandica* **41**, 443-451

De Klerk GJ (2002) Rooting of microcuttings: Theory and practice. *In Vitro Cellular and Developmental Biology – Plant* **38**, 415-422

De Klerk GJ (2007) Stress in plants cultured *in vitro*. *Propagation of Ornamental Plants* **7**, 129-137

De Klerk GJ (2009) Adventitious regeneration. In: Flickinger MC (Ed) *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology* (Vol 1), John Wiley & Sons Inc, Chichester, pp 72-87

De Klerk GJ (2010) Why plants grow in tissue culture? *Prophyta Annual* **2010**, 42-44

De Klerk GJ, Kim KS, Van Schadewijk M, Gerrits M (1992) Growth of bulblets of *Lilium speciosum in vitro* and in soil. *Acta Horticulturae* **325**, 513-520

De Klerk GJ, Paffen A (1995) The effects of environmental conditions on sprouting of micropropagated lily bulblets with various levels of dormancy. *Acta Botanica Neerlandica* **44**, 33-39

De Klerk GJ, Pumisutapon P (2008) Protection of *in-vitro* grown *Arabidopsis* seedlings against abiotic stresses. *Plant Cell, Tissue and Organ Culture* **95**, 149-154

De Klerk GJ, Ter Brugge J (2011) Micropropagation of *Dahlia* in static liquid medium using slow-release tools of medium ingredients. *Scientia Horticul-*

turae **127**, 542-547

Delvallée I, Paffen A, De Klerk GJ (1990) The development of dormancy in bulblets of *Lilium speciosum* generated *in vitro*. 2. The effect of temperature. *Physiologia Plantarum* **80**, 431-436

Eeuwens CJ, Lord S, Donough CR, Rao V, Vallejo G, Nelson S (2002) Effects of tissue culture conditions during embryoid multiplication on the incidence of "mantled" flowering in clonally propagated oil palm. *Plant Cell, Tissue and Organ Culture* **70**, 311-323

Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. *Plant Cell, Tissue and Organ Culture* **69**, 215-231

Evert RF (2006) *Esau's Plant Anatomy* (3rd Edn), John Wiley & Sons, Inc., Hoboken, New Jersey, 384 pp

George EF, Debergh PC (2008) Micropropagation, uses and methods. In: George EF, Hall MA, De Klerk GJ (Eds) *Plant Propagation by Tissue Culture (3rd Edn, Vol 1), The Background*, Springer-Verlag GmbH, Heidelberg, pp 29-64

Ghaffoor A, Imran Maqbool I, Waseem K, Quraishi A (2004) *In vitro* response of tulips (*Tulipa gesnerina* L.) to various growth regulators. *International Journal of Agriculture and Biology* **6**, 1168-1169

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA, Pillot JP, Letisse F, Matusova R, Danoun S, Portais JC, Bouwmeester H, Bécard G, Beveridge CA, Rameau C, Rochange SF (2008) Strigolactone inhibition of shoot branching. *Nature* **455**, 189-194

Grondeau C, Samson R (1994) A review of thermotherapy to free plant materials from pathogens, especially seeds from bacteria. *Critical Reviews in Plant Sciences* **3**, 57-75

Grootaarts H, Schel J, Pierik R (1981) The origin of bulblets formed on excised twin scales of *Nerine bowdenii* W. Watts. *Plant Cell, Tissue and Organ Culture* **1**, 39-46

Gude H, Dijkema MHGE (1997) Somatic embryogenesis in tulip. *Acta Horticulturae* **430**, 275-280

Hol GMGM, Van Der Linde PCG (1992) Reduction of contamination in bulb-explant cultures of *Narcissus* by a hot water treatment. *Plant Cell, Tissue and Organ Culture* **31**, 75-79

Ilczuk A, Winkelmann T, Richartz S, Witomska M, Serek M (2005) *In vitro* propagation of *Hippeastrum* × *chmielii* Chm. - Influence of flurprimidol and the culture in solid or liquid medium and in temporary immersion systems. *Plant Cell, Tissue and Organ Culture* **83**, 339-346

Jackson MB (1985) Ethylene and responses of plants to soil waterlogging and submergence. *Annual Review of Plant Physiology* **36**, 146-174

Kahane R, Rancillac M, De La Serve BT (1992) Long-term multiplication of onion (*Allium cepa* L.) by cyclic shoot regeneration *in vitro*. *Plant Cell, Tissue and Organ Culture* **28**, 281-288

Kamerbeek GA, Beijersbergen JCM, Schenk PK (1970) Dormancy in bulbs, and corms. In: Goren N, Mendel K (Eds) *Proceedings of the XVIIIth International Horticiltural Congress* (Vol 5), Tel Aviv, pp 233-240

Karssen CM, Brinkhorst-Vanderswan DLC, Breekland AE, Koornneef M (1983) Induction of dormancy during seed development by endogenous abscisic-acid - studies on abscisic-acid deficient genotypes of *Arabidopsis thaliana* (L) Heynh. *Planta* **157**, 158-165

Kim KS, Davelaar E, De Klerk GJ (1994) Abscisic acid controls dormancy development and bulb formation in lily plantlets regenerated *in vitro*. *Physiologia Plantarum* **90**, 59-64

Kim KW, De Hertogh AA (1997) Tissue culture of ornamental flowering bulbs (geophytes). *Horticultural Reviews* **18**, 87-169

Koster J (1993) *In vitro* propagation of tulip. Formation and bulbing of shoots on bulb-scale explants from *Tulipa gesneria* L. Thesis, Leiden University, 128 pp

Krikorian AD, Kann RP, O'Connor SA, Fitter MS (1986) Totipotent suspensions as a means of multiplication. In: Zimmerman RH, Griesbach RJ, Hammerschlag FA, Lawson RH (Eds) *Tissue Culture as a Plant Production System for Horticultural Crops*, Martinus Nijhoff, Dordrecht, pp 61-72

Langens-Gerrits MM (2003) Phase change, bulb growth and dormancy development in lily. PhD thesis, University of Nijmegen. 108 pp. http://repository.ubn.ru.nl/bitstream/2066/19304/1/19304_phaschbug.pdf

Langens-Gerrits MM, Nashimoto S (1997) Improved protocol for the propagation of narcissus *in vitro*. *Acta Horticulturae* **430**, 311-314

Langens-Gerrits MM, Albers M, De Klerk GJ (1998) Hot-water treatment before tissue culture reduces initial contamination in *Lilium* and *Acer*. *Plant Cell, Tissue and Organ Culture* **52**, 75-77

Langens-Gerrits MM, De Klerk GJ, Croes AF (2003a) Phase change in lily bulblets regenerated *in vitro*. *Physiologia Plantarum* **119**, 590-597

Langens-Gerrits MM, Miller WBM, Croes AF, De Klerk GJ (2003b) Effect of low temperature on dormancy breaking and growth after planting in lily bulblets regenerated *in vitro*. *Plant Growth Regulation* **40**, 267-275

Leifert C, Waites WM (1992) Bacterial growth in plant tissue culture media. *Journal of Applied Bacteriology* **72**, 460-466

Lilien-Kipnis H, Azizbekova N, Ziv M (1994) Scaled-up proliferation and regeneration of *Nerine* in liquid cultures Part II. Ontogeny of somatic embryos and bulblet regeneration. *Plant Cell, Tissue and Organ Culture* **39**, 117-123

Ongaro V, Leyser O (2008) Hormonal control of shoot branching. *Journal of Experimental Botany* **59**, 67-74

Paffen A, Langens M (2003) Onderzoek Nerine. Aangepaste methode lost slapers probleem op. *Bloembollenvisie* **1 (13)**, 18-19

Podwyszyńska M (2012) The mechanisms of the *in vitro* storage organ formation in ornamental geophytes. In: Van Tuyl J, Arens P (Eds) Bulbous Ornamentals (Bulbs, Cormels and Tubers). *Floriculture and Ornamental Biotechnology* **6 (Special Issue 1)**, 9-23

Prakash J (2009) Micropropagation of ornamental perennials: Progress and problems. *Acta Horticulturae* **812**, 289-294

Schönherr J (2006) Characterization of aqueous pores in plant cuticles and permeation of ionic solutes. *Journal of Experimental Botany* **57**, 2471-2491

Sena G, Birnbaum KD (2010) Built to rebuild, in search of organizing principles in plant regeneration. *Current Opinion in Genetics and Development* **20**, 460-465

Shahin H, Bekhit M, Mohamed-Yasseen Y, Ibrahim AI, De Klerk GJ (2013) Improved micropropagation of white squill (*Urginea maritima*). *In Vitro Cellular and Developmental Biology – Plant* in press

Sharman BC (1945) Leaf and bud Initiation in the Gramineae. *Botanical Gazette* **106**, 269-289

Smulders MJM, De Klerk GJ (2011) Epigenetics in plant tissue culture. *Plant Growth Regulation* **63**, 137-146

Taiz L, Zeiger E (2006) Topic 3.2, Calculating half-times of diffusion. Plant Physiology, Fourth Edition Online. http://5e.plantphys.net/article.php?ch=t&id=26

Tanaka K, Fujiwara K, Kozai T (1992) Effects of relative humidity in the culture vessel on the transpiration and net photosynthetic rates of potato plantlets *in vitro*. *Acta Horticulturae* **319**, 59-64

Topoonyanont N, Debergh PC (2001) Reducing bushiness in micropropagated gerbera. *Plant Cell, Tissue and Organ Culture* **67**, 133-144

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, Kyozuka J, Yamaguchi S (2008) Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**, 195-200

Van Aartrijk J, Blom-Barnhoorn GJ (1984) Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro* interacting effects of NAA, TIBA, wounding, and temperature. *Journal of Plant Physiology* **116**, 409-416

Van Aartrijk J, Van Der Linde PCG (1986) *In vitro* propagation of flowerbulb crops. In: Zimmerman RH, Griesbach RJ, Hammerschlag FA, Lawson RH (Eds) *Tissue Culture as a Plant Production System for Horticultural Crops*, Martinus Nijhoff Publishers, Dordrecht, pp 317-331

Van Bel AJE (1993) Strategies of phloem loading. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 253-281

Van Der Linde PCG, Schipper JA (1992) Micropropagation of iris with special reference to *Iris x hollandica* Tub. Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry (Vol 20) High-Tech and Micropropagation IV*, Springer, Berlin, pp 173-197

Van Harmelen MJ, Löffler HJM, Van Tuyl JM (1997) Somaclonal variation in lily after *in vitro* cultivation. *Acta Horticulturae* **430**, 347-350

Ziv M (1990) Morphogenesis of *Gladiolus* buds in bioreactors for scaled-up propagation of geophytes. In: Nijkamp HJJ,Van Der Plas LHW, Van Aartrijk J (Eds) *Progress in Plant Cellular and Molecular Biology*, Kluwer, Dordrecht, pp 119-124

Ziv M, Lilien-Kipnis H (2000) Bud regeneration from inflorescence explants for rapid propagation of geophytes *in vitro*. *Plant Cell Reports* **19**, 845-850

Ziv M, Kahany S, Lilien-Kipnis H (1994) Scaled-up proliferation and regeneration of *Nerine* in liquid cultures. Part I. The induction and maintenance of proliferating meristematic clusters by paclobutrazol in bioreactors. *Plant Cell, Tissue and Organ Culture* **39**, 109-115