FLOWERING OF GEOPHYTES *IN VITRO*

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Abstract

In vitro flowering is advantageous, especially for species with a long juvenile period like geophytes. The reviewed studies showed that *in vitro* conditions significantly shortened the juvenile period in geophytes. Media components, level of plant growth regulators (PGR), and culture conditions affect *in vitro* flowering, as does the physiological stage of the mother plant and the organ or tissue source of the explant. Thus, *in vitro* conditions can be manipulated and provide an alternative controlled system necessary to study flower induction, as well as inflorescence and flower morphogenesis. It can also be applied as a tool to accelerate breeding programs or can be adjusted to commercial production of specific compounds from floral organs. The levels and ratio of promoting and retarding PGR in the medium were found to be strongly correlated with the initiation and development of floral organs from buds or callus tissue. Recently, PGR levels were shown to affect gene regulation of floral organ development *in vitro*. This review covers various aspects of controlling flower induction and development of geophytes *in vitro*. The review emphasizes the importance of the tissue culture system as a tool to study various aspects of flowering control for the potential advancement of geophytes in the ornamental, food, medicinal, and craft industries.

Key words: Auxin, cytokinin, explant origin, flowering, geophytes, gibberellin, tissue culture

INTRODUCTION

The reproductive stage, or flowering process, is one of the critically important stages in plant development and is vital for the completion of the life cycle and seed production. This process involving the induction and development of flowers and their organs has been studied and manipulated for a large number of plants *in vitro*. The advances in cell and tissue culture techniques provide an excellent system for studying the physiological and molecular biology aspects of plant science, including flowering.

This review focuses on *in vitro* flowering in geophytes. Many geophytes have an important commercial value in the ornamental, food, medicinal, and craft industries. Breeding programs are constantly being used to improve the quality traits of these plants. However, their long juvenile period prolongs breeding programs while the use of mutants for research is practically impossible. *In vitro* tissue culture techniques for flowering can be an important tool to achieve these goals. The system involves the culture of isolated, aseptic plant sections (explants) or organs, such as roots, leaves,

buds, and flowers in sterile containers with a defined medium under controlled environmental conditions (Ziv and Altman 2003).

To study various aspects of flowering, isolated vegetative or reproductive buds were cultured in media containing various combinations of inorganic and organic components and plant growth regulators (PGR). The aim of these studies was to induce and control the process of flower formation in plants. The studies were carried out with plants having different photoperiodic or thermoperiodic requirements, and the explants in these studies were isolated at different physiological stages. In addition, thin cell layer (TCL) explants or tissue from vegetative or reproductive organs of juvenile or mature plants were also used for the study of *in vitro* flowering (Tran Thanh Van 1999). Nevertheless, flowering *in vitro* is not a widespread phenomenon. It occurs spontaneously or deliberately in several herbaceous and geophyte species (de Fossard 1974, Scorza 1982, Dickens and van Staden 1988, Rastogi and Sawheny 1989, Taixeira da Silva 2003). Deliberate flowering in culture can serve as a tool for studying flower induc-

tion and development and for controlling breeding programs in species with long juvenile periods, e.g., geophytes (Lin et al. 2003b). Another application of *in vitro* flowering is for metabolite production as in *Crocus*, where flowering *in vitro* is advantageous as a source of commercial production of stigmas with aromatic and pigmented compounds (Sano and Himeno 1987, Plessner and Ziv 1999). Direct amplification of reproductive organs in protocols, which were developed for ginseng and bamboo, can serve as a source for medicinal compound production (Lin et al. 2003a). In contrast, *in vitro* flowering in other geophytic species can be undesirable as in the commercial production of monocarpic species of bamboo plantlets (Chambers et al. 1991) and *Ornithogalum dubium*. In our recent research with *Ornithogalum dubium*, the developing reproductive buds decreased the rate of vegetative bud propagation. Geophytes are perennial plant species with an underground storage organ and renewal buds. They propagate and survive not only by seeds but also by specialized underground storage organs such as bulbs, corms, tubers, or rhizomes (Raunkiaer 1934). With known reservation, we have included some of the orchids having pseudobulbs and the monocarpic perennial bamboos into this definition. The primary function of the storage organ is to store metabolites and moisture for plant growth and to ensure survival during seasons of limited environmental growth conditions such as low/high temperatures or water stress. Storage organs, categorized according to their morphological structure, are usually divided into bulbous and tuberous groups (De Hertogh and Le Nard 1993). Geophyte genera include both mono- and dicotyledonous species. According to the climatic origin and environmental conditions, winter or summer dormancy mechanisms were developed in geophytes in order to survive adverse conditions. These mechanisms include underground storage organs, winter or summer dormancy and the perception of environmental signals of the seasonal changes. Flower induction in many geophytes is controlled environmentally by photoperiod, temperature, or both (Le Nard and De Hertogh 1993). In other species, flowering is controlled by autonomous signals. In a literature review, Hartsema (1961) divided geophytes into 7 groups of different flower initiation times in relation to the developmental stage of the plants. The ultimate understanding of plant patterns of development is essential for the study of flower induction and development *in vitro*.

Phase changes

Angiosperms pass through three phases in their life cycle: juvenile (non-competent), vegetative adult (competent but not induced), and reproductive (competent and induced) (Poethig 1990, Simpson et al. 1999). The duration of each phase varies from a very short period to a very long one that can last for many years (Scorza 1982). Herbaceous annual plants usually have a very short juvenile period (if at all), and are considered competent for flowering during their growth period. In contrast, in perennial herbaceous plants, including geophytes, and in woody plants, the juvenile stage is clearly distinguished from the adult stage. In geophytes, juvenility (i.e., the inability to flower under inductive conditions) usually lasts 1-3 years, although species with longer periods are known: *Urginea* (Dafni et al. 1981), *Bambusa* (Nadgauda et al. 1990), and tulip (Fortanier 1973, Le Nard and De Hertogh 1993). The switch from juvenility to vegetative adult stage is controlled by endogenous systems (Simpson et al. 1999). Physiologically, the change from juvenile to adult plant is expressed as a change from non-competency to competency (Poethig 1990). Competent meristems are responsive to environmental or autonomous signals that eventually lead to flower formation. The flowering process is expressed by changes in the morphology and developmental physiology of the bud meristem (Simpson et al. 1999). Eventually, after the completion of floral organ formation, in most cases these changes are followed by termination of meristemal activity (Poethig 1990). In geophytes, juvenility is expressed as an undersized storage organ for flowering and/or an undersized bud meristem (Halevy 1990, Langens-Gerrits et al. 2003). In some species, juvenility is expressed by the number of leaves under a critical threshold (Le Nard and De Hertogh 1993, Langens-Gerrits et al. 2003). Minimal bulb size for flowering is genus- or species-dependent and also varies with cultivar and environmental conditions (Le Nard and De Hertogh 1993). However, Langens-Gerrits et al. (2003) showed that in *Lilium* plantlets grown *in vitro*, the physiological age contributed to the change from juvenile to adult phase more than did bulb size. Recent studies in *Zantedeschia* reported *in vitro* flowering of buds in response to GA_3 application. The authors showed that the bud, except for buds in clusters that were cultured on a high level N^6-benzyl adenine (BA) medium, is competent for flowering regardless of its size, position, or age (Naor et al. 2005). Juvenility in geophytes is characterstic of both new tubers or bulbs developing from seeds, and daughter tubers or bulbs developing vegetatively from mature plants. Micro-tubers or bulbs from tissue culture (TC) are also juvenile. Thus, in geophytes reversing from the adult phase to juvenility and loss of competence to flower occurs under natural conditions and in TC (Chang and Hsing 1980, Nadgauda et al. 1990), in contrast to woody plants (Poethig 1990). However, the juvenile phase of *Lilium* bulblets regenerated in TC was much shorter compared to those cultured under natural conditions (Langens-Gerrits et al. 2003). Reversion from reproductive tissue to the formation of vegetative buds was used for micropropagation of various geo-

phytes such as *Allium ampeloprasum, Dichelostemma multiflorum, Eucrosia radiate, Gladiolus grandiflorus, Haemanthus coccineus, Hyacinthus orientalis, Narcissus tazetta, Nerine sariniensis, Ornithogalum dubium*, (Ziv and Lillien-Kipnis 1997, 2000), as well as *Allium cepa* (Keller 1990), *A. sativum* (Xu et al. 2005), and *A. ampeloprasum* (Ziv et al. 1983), *Amaryllis* (Bapat and Narayanaswamy 1976), bamboo (Prutpongse and Gavinlertvatana 1992), *Iris enstata* (Kawase et al.1995, Boltenkov and Zarembo 2005), *Iris setosa* and *Iris sanguinea* (Boltenkov and Zarembo 2005). In these studies flower organs or peduncle and pedicle segments were used as explant sources. This contrasts to buds *in planta* where it can be extremely difficult to reverse the growth to a vegetative phase, once the shoot is fully committed to the new reproductive phase (Rastogi and Sawheny 1989, Poethig 1990). In many species, commitment to flowering can be transferred from the mother plant to plantlets grown in TC (Dickens and Van Staden 1988, Daksha et al. 1994).

Plant organ source for tissue culture (various developmental phases of the bud)

The choice of the source of the explant from the mother plant is determined according to the goals of the study and the ability of the tissue to regenerate buds directly or indirectly via a callus. The explant can be an organ, part of an organ, a tissue (including TCL), or cells. However, as stated by Dickens and Van Staden (1988), in order to study flower induction, the explant should originate from juvenile plants or seeds, while floral development studies require explants from adult and induced plants, like flower buds, floral organs, sections from the flower, or inflorescence stalks. In geophytes the juvenile stage is clearly distinguished from the adult stage. Therefore, the choice of the mother plant is relatively simple and must be considered according to the aims of the study. The ability to regenerate floral organs, probably through transport of the flowering stimulus/stimuli, was proven to be transferred through consecutive sub-culturing. In some species this phenomenon lasted for many years (e.g., 22 years in *Cymbidium*, Kostenyuk et al. 1999). In contrast, flowering *in vitro* faded very fast after one subculture in *Passiflora*, and four subcultures in *Saccharum* spp. (Scorza 1982). However, in carnation plantlets grown in TC, the production of adventitious flowering shoots continued for many subcultures over more than two years (Daksha et al. 1994).

In vitro conditions

The advantage of tissue culture techniques is in their ability to control environmental conditions as well as media components like nutrients, vitamins, promoting and retarding PGR. Media components, growth conditions, and the source of explant affect the morphological response. Early studies on *Aquilegia* (Tepfer et al. 1963, summarized in Rastogi and Sawheny 1989, Tepfer et al. 1966) as well as recent studies in *Hyacynthus* (Lu et al. 1988, 2000) showed that different media components are required to promote the development of different floral organs. Thus, the developmental pattern is dependent on a multi-factor system and any change in one of its components might change the morphogenetic expression. In spite of the generalization for various components, functions and activities, specific requirements (e.g., PGR levels and ratios, nutritional requirements, pH, temperature and light) must be studied separately for each given species or even cultivar (Rastogi and Sawheny 1989).

C/N ratio and mineral levels

Tissue culture media contain mineral and sugar sources in addition to PGR and other organic constituents. It was found that *in vitro* flowering was also influenced by the levels and ratios of the two major components, carbohydrates and minerals. High concentrations of nitrogen in MS media inhibited flowering and promoted vegetative growth, which competed more efficiently for carbohydrates from the medium (Dielen 2001). According to the floral nutrient diversion hypothesis, C/N ratios increase in buds during flower induction (Sachs 1977). Thus, using half-strength MS mineral medium (Murashige and Skoog 1962) or reduced nitrogen level enhanced *in vitro* flowering in geophytes like *Bambusa vulgaris, Dendrocalamus giganteus, D. strictus* (Rout and Das 1994), *Cymbidium* (Kostenyuk et al. 1999), *Doritis* (Duan and Yazawa 1994), ginseng (Chang and Hsing 1980), and in annual herbaceous species like *Orichophragmus violaceus* (Luo and Lan 2000) and tomato (Dielen et al. 2001). In contrast, no difference in the rate of *in vitro* flowering of *Bambusa edulis* plantlets was observed as a result of various concentrations of sucrose and nitrogen in the media (Lin et al. 2003b). High phosphorous in the medium was found to stimulate flowering in *Cymbidium* (Kostenyuk et al. 1999) and *Ornithogalum dubium* (Fig. 1 A, B).

Plant Growth Regulators and flowering in vitro

The role of PGR in regenerating flowers and floral organ development *in vitro* is discussed widely in earlier reviews (de Fossard 1974, Scorza 1982, Dickens and Van Staden 1988, Rastogi and Sawheny 1989, Taixeira da Silva 2003, Naor et al. 2004). Most commonly used PGR for flowering *in vitro* are cytokinins, gibberellins, and auxins. In some species GA_3 and IAA inhibit or reduce flowering, while in other species they were shown as enhancers (de Fossard 1974, Scorza 1982, Dickens and Van Staden 1988, Naor et al. 2004). Earlier studies showed correlations between PGR concentration and morphological changes in relation to flowering (Tepfer et al. 1966, Bilderback 1972, Sano and Himeno 1987,
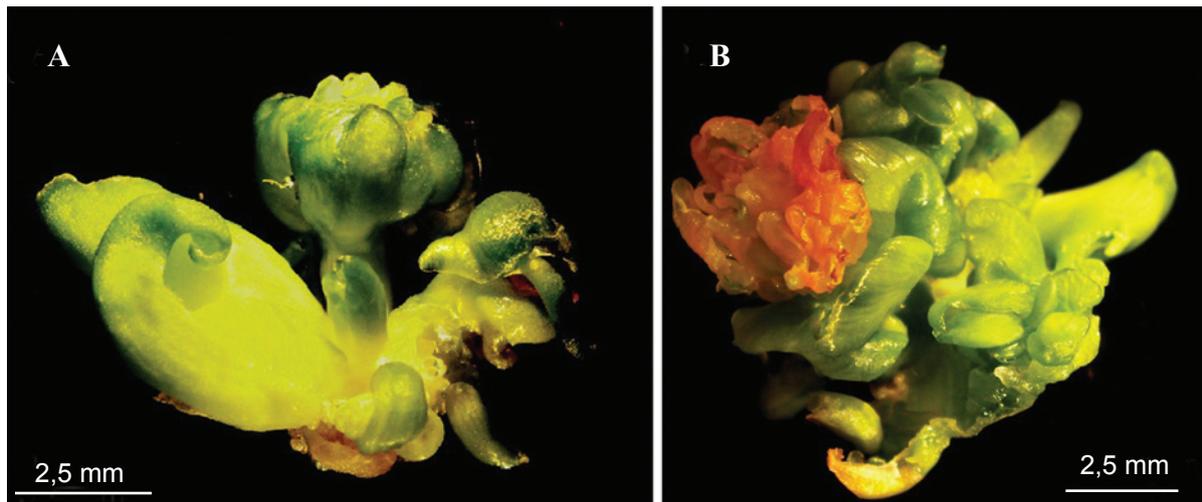
Fig. 1. A) Development of an inflorescence in buds of *Ornithogalum dubium* regenerated from inflorescence stem leaf. Explants subcultured to MS medium enriched with Phosphate and supplemented with 0.54 μM NAA and 8.9 μM BA in the medium. B) An *Ornithogalum dubium* cluster of several shoots with a flowering inflorescence lacking floret peduncles after 6 weeks in culture (explant and medium as in Fig 1A).

Lu et al. 1988, Lee et al. 1990, 1991, summarized in Dickens and Van Staden 1988, Duan and Yazawa 1994, Kostenyuk et al. 1999, Lu et al. 2000, Luo and Lan 2000, Tang 2000, Chang and Chang 2003, Lin et al. 2003b, 2004, 2005). Using molecular biology tools, recent studies in *Hyacinthus* proved a causal relationship between PGR and floral organ development (Li et al. 2002, Xu et al. 2004). It seems that changes in PGR ratios are necessary for the development of different floral whorls or organs. Table 1 summarizes the involvement of various PGR in flowering of geophytes *in vitro*. When the explant is a floral bud or organ it is clear that PGR can affect organ morphogenesis and development of various flower organs. However, when the explant is a vegetative bud or tissue, PGR can affect the flower induction phase, the floral organ development, or both. In some cases *in vitro* flowering occurred autonomously on a PGR-free medium (Table 1), apparently as a response to endogenous relations between PGR and other physiological factors in the bud.

Gibberellin enhanced *in vitro* inflorescence development in *Zantedeschia* similarly to its effect *in vivo* (Naor et al. 2004). Gibberellin and cytokinins stimulated flowering *in vitro* in *Orichophragmus violaceus* and apparently substituted cold treatment (Luo and Lan 2000).

In vitro flower morphology

In vitro flowers and inflorescences are often smaller than normal *in vivo* ones (Rastogi and Sawheny 1989, Nadgauda et al. 1990, 1997, Chang and Chang 2003, Naor et al. 2004) while normal-sized flowers developed in *Cymbidium* (Tran Thanh Van 1974) with color pigments (Kostenyuk et al. 1999). In some cases whorl

development is incomplete (Tepfer et al. 1966, Lin et al. 2003a) or malformed (Scorza 1982). However, viable pollen and seed set were reported for bamboo and *Cymbidium* (Nadgauda et al. 1990, 1997, Chang and Chang 2003). The pattern and order of whorl development is usually similar to *in vivo* patterns but the level of development often depended on the explant source and PGR levels in the medium. This is also the case for the pattern of inflorescence development as seen in the spikelet development of *Bamboosa arundinacea* Willd and *Dendrocalamus brandisii Kurz.* (Nadgauda et al. 1990, 1997). In colored *Zantedeschia* spp grown in TC, male florets were well distinguished from female florets on the spadix of the miniature inflorescence (Fig. 2A, Naor et al. 2004). Controlled conditions enabled indeterminate formation of specific organs (Lu et al. 1988, 2000) and amplification of inflorescences (Lin et al. 2003a, 2004) in response to changes in PGR levels and ratios (Scorza 1982).

In vitro flowering in geophyte species

Allium

In vitro flowering was reported for *Allium sativum* (Tizio 1979) and *Allium ampeloprasum* (Ziv et al. 1983, Ziv and Lillien-Kipnis 2000). Segments of the floral stalk and inflorescence meristem at the early stage of differentiation were used as explant sources, respectively. In *Allium sativum* floret formation competed with aerial bulb growth in the presence of 10^{-5} M GA_3 and adenine or biotin in the medium, whereas adenine antagonized GA_3 and enhanced bulblet formation (Tizio 1979). Floral buds of *A. ampeloprasum* were formed from inflorescence meristems at early stages of reproductive organ differentiation in

Table 1. The effects of plant growth regulators on flowering *in vitro*.

Species	Response	Origin of explant	Gibberellin	Cytokinin	Auxin	References
Developmental response on media with PGR						
Allium sativum	Florets compete with bulblets	Inflorescence stalk	[1]GA$_3$+	KN-		Tizio 1979
Allium ampeloprasum	Floral buds	Early differentiation of reproductive organs		BA+	NAA+	Ziv and Lilien-Kipnis 2000
Bambusa vulgaris, Dendrocalamus giganteus, D. strictus	Spikelets with flowers and seeds	Seeds	[2]GA$_3$+		IBA+	Rout and Das 1994
B .arundinacea, D. brandisii	Spikelets with flowers	Seeds		[3]BA+, CM+		Nadgauda et al. 1990, 1997
B. edulis	Spikelets with flowers; Spikelets amplification	Spikelet, root of adult phase plant	GA$_3$-	[4]TDZ+	NAA only+, NAA negative interaction with TDZ	Lin et al. 2003a, b, 2004
Cichorium intybus	Flowers	Seeds	GA$_3$+			Bais et al. 2000
Crocus sativus	Flower-like organs	Floral tissue, flower stalk, corm sections, meristem, callus		BA usually +	NAA usually +	Sano and Himeno 1987, Plessner and Ziv 1999
Cymbidium spp.	Inflorescence with flowers	Rhizome from juvenile plant 22 years in culture, callus of unknown origin, pseudo-bulbs unknown physiological age		+	No clear effect of IAA	Tran Thanh Van 1974, Kostenyuk et al. 1999, Chang and Chang 2003
Doritis pulcherimma x Kingiella philipinensis	Flower	Flower stalks		+		Duan and Yazawa, 1994,1995b
Hyacinthus orientalis	Flower and flower organs	Floral organs		BA+	2,4-D +	Lu et al. 1988, Li et al. 2000, 2002, Xu et al. 2004
Panax ginseng	Flowers	Seeds	GA$_3$+?	BA/TDZ + interact with GA$_3$	High dose -	Chang and Hsing 1980, Lee et al. 1990, 1991, Tang 2000, Lin et al. 2005
Kniphofia leucocephala	Inflorescence with immature florets	Lateral meristems		BA+		Taylor et al. 2005
Zantedechia spp.	Inflorescence with incomplete male and female florets	Vegetative buds from tubers	(GA$_1$, GA$_3$, GA$_4$) +	(BA interacts with GA$_3$)		Naor et al. 2004
Developmental response on PGR-free media (using environmental induction)						
Cichorium intybus	Flowers	Root tissue of unknown plant phase, vernalized root explants				Demeulemeester et al. 1995b, Demeulemeester and De Proft 1999
Dendrocalamus. hamiltonii	Spikelets with flowers	Seeds		4 weeks on free-PGR medium after 8 weeks with BA		Chambers et al. 1991
Iris	Flower	Bulb shoot apex				Rodrigues Pereira 1961
Lilium x cv. Star Gazer	Phase change from juvenility to adult buds	Scales from bulbs of unknown phase				Langens-Gerrits et al. 2003
Ornithogalum arabicum	Inflorescence	Scale tissue from mature bulb				Halaban et al. 1965

[1] +: a positive effect, -: a negative effect, ?: the effect is not clear.
[2] Adenine was also added
[3] CM- coconut milk.
[4] Kinetin, BA, Zeatin, PBA (n-benzyl-9-(2-tetrahydropropanyl)-adenine and adenine were less effective.
Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - N^6-benzyl adenine; CM - coconut milk, DNP - day neutral plant; GA - gibberellic acid; IAA - indole acetic acid; IBA - Indole-3-butyric acid; KN - kinetin; NAA - α-naphthalene acetic acid; PGR - plant growth regulators; TC - tissue culture; TCL - thin cell layer.

the presence of 5.4 μM NAA and 25.0 μM BA in the medium. In these explants, reversion to vegetative bud formation was unsuccessful (Ziv and Lillien-Kipnis 2000). Vegetative buds developed from segments of inflorescence stalks (*A. ampeloprasum*, Ziv et al. 1983), and from meristematic tissues located between the floral pedicles on the capitulum (*A. ampeloprasum*, Ziv and Lillien-Kipnis 2000, *A. sativum*, Xu et al. 2005).

Bambusa spp. (bamboo)

This genus can be considered as belonging to the group of geophytes because the plants are perennial, the culmous stalk of the temperate species develops from the rhizome, and the rhizome contains starch as a storage compound. The rhizome acts as a transient storage organ, for the transfer of assimilates from old to newly developing culms (Kleinhenz and Midmore 2001). We considered this genus as a geophyte with some reservation due to the monocarpic pattern of its development. Flowering in nature occurs in laps of 2-120 years (Nadgauda et al. 1990, Chambers et al. 1991, Kleinhenz and Midmore 2001, Lin et al. 2003b). In bamboos, in contrast to other grasses, the glumes of the pseudo-spikelets subtend dormant buds that can develop into new pseudo-spikelets. This phenomenon is used for indeterminate development of pseudo-spikelets in TC where cytokinin was found to be inductive (Gieles et al. 2002). Bamboos are considered monocarpic species and most of them display a gregarious habit of growth (Chambers et al. 1991, Prutpongse and Gavinlertvatana 1992, John and Nedgauda 2001, Kleinhenz and Midmore 2001). Gregarious plants from the same local population or from the same clonal origin flower more or less at the same time and die after one flowering season (Nadgauda et al. 1990, Kleinhenz and Midmore 2001). This suggests that either flowering is autonomously controlled (Nadgauda et al. 1997) or is controlled by multiple, but unpredictable environmental signals (Nadgauda et al. 1990). These reproductive characteristics prevent controlled breeding (Nadgauda et al. 1990, 1997) but can be overcome by *in vitro* flowering (Gieles et al. 2002). On the other hand, programmed death was observed in shoots that flowered *in vitro*, imposing a problem when tissue culture is used for commercial mass propagation (Lin et al. 2003b). However, Gieles et al. (2002) reported a vegetative growth of plants *ex vitro* from pseudo-spikelet-derived plantlets of three species of bamboo. *In vitro* flowering occurred in seedlings of seven species: *Bambusa arundinacea* (Nadgauda et al. 1990, 1997), *Dendrocalamus brandisii* (Nadgauda et al. 1990), *Dendrocalamus brandisii* (Chambers et al. 1991), *Bambusa vulgaris, Dendrocalamus giganteus, Dendrocalamus strictus* (Rout and Das 1994), *Bambusa edulis* (Lin et al. 2003b). Prutpongse and Gavinlertvatana (1992) report a sporadic and low rate of *in vitro* flowering in

B. arundinacea, B. brandisii, B. glaucescens, B. multiplex, B. nana, B. spp. 'Dam Khan'*, Cephalostachym pergracile and D. membranaceus*. For six species *in vitro* flowering occurred either from cultured seeds that developed into seedlings, or from plantlets derived from zygotic seedlings (via callus) after ca. 2-3 subcultures (Nadgauda et al. 1990, 1997, Chambers et al. 1991), or from nodal explants derived from somatic embryos (Rout and Das 1994). In one of the species, flowering developed *in vitro* from inflorescence segments. Flowers were observed 12-15 weeks after seed sowing or explant subculture, thus shortening the juvenile period considerably. In most studies, BA was the most common cytokinin used in the growing media, while TDZ was found to be the most effective cytokinin (Lin et al. 2003b). Coconut milk was not an essential component. Amplification of inflorescences resulted after 10 months of repeated subculturing on MS media supplemented with 0.5-4.5 μM TDZ (Lin et al. 2003a, 2004). However, Rout and Das (1994) reported *in vitro* flowering of three species on MS medium supplemented with 1.2 μM IBA, 1.2 μM adenine sulphate and 1.4 μM GA$_3$. Nevertheless, auxin was shown to be antagonistic to cytokinin in spikelet development and inflorescence amplification (Lin et al. 2003b, 2004). Floret size was smaller than *in planta*, however, in five species fertile seeds were produced (Nadgauda et al. 1990, Rout and Das 1994, Nadgauda et al. 1997). *In vitro* flowering studies in *Bambusa* have a vital role for understanding the physiology of monocarpic plants.

Cichorium intibus (chicory)

While *Cichorium intibus* is a biennial plant, there are also several perennial species. We thus considered it with some reservation as having geophytic traits, because the storage root bears renewal buds for the second year of growth. Chicory plants require both long days and a cold period for flowering (Demeulemeester et al. 1995b). Plantlets derived from vernalized root tissue or seedlings flowered spontaneously *in vitro* (Demeulemeester et al. 1995a, Bais et al. 2000). An attempt to substitute the vernalization requirements for inflorescence initiation and development in TC with exogenous application of 5-azacytidine, a DNA demethylation agent, resulted in only 15% flowering (Demeulemeester and De Proft 1999). In another study, putrescine, a precursor of the polyamines spermine and spermidine, was found to be involved in flower development *in vitro* when 40 mM were added along with 14.4 μM GA$_3$ to the medium. Its role in flower development was shown by exogenous application or by the effect of the putrescine biosynthesis inhibitors di-flouro-methyl-ornithine (DFMO) and di-flouro-methyl-arginine (DFMA), as well as by the application of AgNO$_3$. S-adenosyl methionine (SAM) is a common precursor of ethylene and putrescine. Thus AgNO$_3$, which inhibits

ethylene action, increased *in vitro* flowering possibly via increased putrescine production (Bais et al. 2000). Inhibitors of gibberellin biosynthesis suppressed flower stem growth but not inflorescence differentiation in chicory plantlets *in vitro*. It was proposed that GA_1 is synthesized during *in vitro* culture and controls floral stem elongation but not inflorescence differentiation (Demeulemeester et al. 1995a). *In vitro* explants were found to be competent for flowering at all bud stages in contrast to plants *in vivo* (Demeulemeester and De Proft 1999). It was suggested that an unknown flowering inhibition factor was removed by excising the explants and culturing them *in vitro* (Demeulemeester et al. 1995a, Demeulemeester and De Proft 1999), or by growing the explants under at least a 16-h photoperiod (Demeulemeester et al. 1995b).

Corydalis yanhusuo

In vitro mass propagation is advantageous for medicinal herb plants (Kuo et al. 2002). The tubers of *Corydalis*, an important herb in the Chinese medicine, contain several pharmaceutically important alkaloids. Kuo et al. (2002) examined *in vitro* plant regeneration from somatic embryos that differentiated from tuber callus. *In vitro* flowering of well developed plantlets was observed after four months in culture. The physiological stage of the mother plant tissue was not studied, however. The *in vitro* flowering rate was very low (5%), and the PGR effect on flowering *in vitro* was not clear.

Crocus sativus (Saffron crocus)

The aim of *in vitro* flowering of *Crocus sativus* L. was to induce flower development for the commercial production of stigmas, which contains valuable compounds used in the spice industry (Sano and Himeno 1987, Plessner and Ziv 1999). Pigments and aromatic compounds in the stigma make it a precious spice in oriental and western culinary practice. Previous research showed that flower-like structures were produced *in vitro* mostly from floral organs, except in one case where a bud or meristematic tissue was used. In most cases a combination of auxin (usually NAA) and cytokinin (usually BA) was effective in one of the following media: MS (Murashige and Skoog 1962)**,** W (White 1963), LS (Linsmaier and Skoog 1965), N6 (Nitsch and Nitsch 1969), B5 (Gambourg et al. 1968), supplemented with sucrose or coconut milk (Plessner and Ziv 1999). These PGR were used for the regeneration of the fourth whorl, i.e., the pistil with the stigma. Increased formation of pigmented stigma-like structures *in vitro* was reported when the medium was supplemented with auxin (NAA or IBA) together with the cytokinins kinetin or BA (Sano and Himeno 1987), or with elevated sucrose levels together with BA, NAA, and alanine (Otsuka et al. 1992). The quality and quantity of saffron compounds depended on the type of tissue or organs regenerated *in vitro* (Plessner and Ziv 1999). Stigma-like structures from various flower organs contained low levels of aromatic pigments (Sano and Himeno 1987), or even different pigments that are normally found in plants under natural conditions. However, in some cases the composition and quantity of pigments and saffron compounds were similar to the natural substances *in planta* (Sano and Himeno 1987).

Hyacinthus orientalis (hyacinth)

Regeneration of flower organs and complete flowers of *Hyacinthus* is possible from segments of the perianth (Lu et al. 1988). Direct organogenesis was observed within 50-200 days in culture, providing a system to study factors controlling flower development. The *Hyacinthus* flower develops in four consecutive whorls. The switch from one whorl to another is genetically controlled and mediated via changes in PGR levels. In *Hyacinthus* high levels of BA (8.9 µM) and auxin (2,4-D, 0.5 µM**)** resulted in indeterminate tepal development *in vitro*. A high level of BA (8.9 µM) and low level of auxin (2,4-D 0.5 µM) induced stamen (Lu et al. 2000) and carpel (Lu et al. 1988) development. The authors showed that the phytohormone control of floral organ development is strictly concentration-dependent. When there was no change in the hormone concentration, the specific floral organ differentiated indeterminately producing over 100 tepals, 20 stamens, or 40 ovules (Lu et al. 1988, 2000). Using *in vitro* methods it was shown that changes in PGR levels switch on and off the *HAG1* and *HoMADS1* genes of the ABCD model of floral organ development (Li et al. 2002, Xu et al. 2004).

Iris

Wedgwood iris is a monocotyledonous day-neutral plant (Rodrigues Pereira 1961). Under natural conditions, the floral stem develops within the bulb after planting during the cold season (De Munk and Schipper 1993), or when stored at 13ºC for a specific time period (Hartsema 1961). When stored at 25.5°C, the apex remained vegetative for over 10 months. However, during this period the apex was induced, and when transferred to 13°C, 60% of the buds became floral (Rodrigues Pereira 1961). Ethylene was found to enhance flower differentiation in induced undersized bulbs (Imanishi et al 1994). Using TC, Rodrigues Pereira (1961) examined the environmental signals that switch the apex from a vegetative to a reproductive phase. He correlated the signals with endogenous changes in gibberellin-like substances that might serve as internal mediators. *In vitro* flowering in the apices of wedgwood iris bulbs given various periods of storage at 25.5 and 13ºC showed that phase change occurred if inductive treatments were given prior to bud excision. Inductive conditions depended on a minimal period at 25.5ºC, if

followed by one week of storage at 13ºC. However, a longer storage duration at 13ºC could not compensate for reducing the storage period at 25ºC in order to obtain flowering *in vitro*. The external signal responsible for the phase change in the bud was mediated by factors present in the scales. Therefore, induced scales isolated from the shoot apex and placed on the medium surface, or bud extracts in the medium increased the rate of floral apices formation**,** showing that the induction signal was transferred from the scales to the apex. In contrast, purple perianth lobe-like organs were formed from calli derived from flower buds of *Iris enstata* regardless of PGR levels in the media. This phenomenon lasted for two years of consecutive subculturing (Boltenkov and Zarembo 2005).

Kniphofia leucocephala

In vitro flowering of *Kniphofia leucocephala* (Asphodelaceae), an endangered species in South Africa, was observed from lateral buds on MS supplemented with PGR. Relatively high levels of BA (35.5 µM) promoted inflorescence differentiation in 30% of the shoots (McCarten and Van Staden 2003). The florets were immature (no reproductive organs), malformed, and non-pigmented, and eventually turned green and senesced. In the presence of cytokinin, inflorescence differentiation was observed under long-day, short-day or day-neutral regimes. Among three cytokinins tested, BA gave the highest rate of inflorescences (60%) when 20 µM were supplemented to the medium. Delaying floret abortion with GA_3 was unsuccessful emphasizing the importance of the time of application. The authors suggest an interaction between sucrose and BA to promote flowering in *Kniphofia* (Taylor et al. 2005).

Lilium (Lilium x cv. Star Gazer)

In vitro flowering was not achieved in *Lilium*. However, a phase change from juvenility to the adult phase was reported (Langens-Gerrits et al. 2003). There are three phases in the lily plant cycle: a juvenile phase, where one or a few leaves with swollen petioles develop, a vegetative adult bulb phase, with a stem and stretched internodes and many leaves, and a flowering phase, when the terminal inflorescence develops. Under natural conditions, juvenility is correlated with bulb size. Thus, the transition from the juvenile to the adult phase when the meristem is competent, usually takes 1-2 seasons. However, Langens-Gerrits et al. (2003) showed that bulblets grown in TC for 12 weeks at 25ºC followed by 2-4 weeks at 15ºC became competent after a considerably shorter period. This transition was characterized by increased cell divisions and the formation of a tunica-corpus structure in the apical meristem. Factors like explant size and sucrose concentration, which stimulated bulb growth and size, also enhanced the phase change. On the other hand, low phosphorous

concentration enhanced a phase change regardless of bulblet size. The physiological state of the bulb contributed to the phase change more than the bulb size. Thus, in this study factors involved in meristem competence were studied. These results can contribute to studies of the endogenous changes of PGR and genetic systems in the apical meristem that precede flowering *in vivo* and *in vitro*.

Orchids

Many orchids are considered geophytes because of their rhizome or their pseudobulb acting as a storage organ. *In vitro* flowering in orchids was reported in several studies, including four geophytic species (Duan and Yazawa 1994, 1995b, Kostenyuk et al. 1999, Chang and Chang 2003). It takes 3-13 years to produce a flowering plant from seeds, thereby making breeding programs very slow (Duan and Yazawa 1995b, Kostenyuk et al. 1999, Chang and Chang 2003). The long juvenile period can be shortened significantly by tissue culture micropropagation (Duan and Yazawa 1994, Yu and Goh 2001). *In vitro* systems are used to study the physiological and molecular basis of the orchid flowering process, to hasten flowering in slow-maturing orchids and to improve the control of forcing and flower-timing (Mudalige and Kuehnle 2004). The molecular basis of flowering in orchids was reviewed recently by Yu and Goh (2001) and Mudalige and Kuehnle (2004). Seventy genes have been cloned so far from 7 genera of which some relate to flowering. The profile of gene expression during the transition to flowering has been established in *Dendrobium* spp. using an *in vitro* flowering system (Yu and Goh 2000).

Cymbidium spp.

In nature, the juvenile period of *Cymbidium niveomarginatum* and *C. ensifolium* var. *misericors* lasts 4-7 years. Studies showed *in vitro* flowering after 40-100 days from the last sub-cultured plants (Kostenyuk et al. 1999, Chang and Chang 2003). This phenomenon was shown in explants of *C. niveo-marginatum* used through 22 consecutive years of sub-culturing (Kostenyuk et al. 1999) or from rhizome-derived calli of *C. ensifolium* var. *misericors* (Chang and Chang 2003). The *in vitro* systems were used to study the role of PGR in flower initiation and development. Since *Cymbidium* orchids are considered day neutral plants (DNP) (Dole and Wilkins 2004), understanding the role of PGR is essential for the study of genetic control for plant improvement (Kostenyuk et al. 1999). *C. niveo-marginatum* responded to cytokinin and auxins differently than did *C. ensifolium* var. *misericors*. *In vitro* flowering occurred in these species in the presence of NAA and different cytokinins. In *C. niveo-marginatum* 44.4 µM BA gave the best results (Kostenyuk et al. 1999), while the inflorescences of *C. ensifolium* var. *misericors* developed in the presence of

10 μM 2iP (N⁶-(2-isopentenyl)adenine), but not with BA in the medium (Chang and Chang 2003). No inflorescences were observed in the presence of NAA alone in the medium (Chang and Chang 2003, Kostenyuk et al. 1999), and thus the auxin role in flower initiation and development is not clear. TDZ was effective for flower initiation in both species. However, it resulted in poor plant growth of *C. niveo-marginatum* and the flower buds withered after a short period (Kostenyuk et al. 1999). The differences in responses can be related to the species, the origin of explants, or the physiological stage of the mother plant. The positive response to cytokinin was in accordance with other reports of early flowering in orchids when a high level of cytokinin was used (Duan and Yazawa 1995a). However, in these species it is not clear whether cytokinin is necessary for flower induction or flower development. (Kostenyuk et al.1999). It is also suggested that GA₃ interacts with other PGR in flower induction and development of *C. niveo-marginatum*. Reduced levels of nitrogen promoted *in vitro* flowering of *C. niveo-marginatum* in the presence of BA in the medium. Phosphorous supply provided favorable conditions for *in vitro* flowering of *Cymbidium* (Kostenyuk et al. 1999, Chang and Chang 2003). The flowers of *C. ensifolium* var. *misericors* were three times smaller than flowers *in vivo* but structurally normal and produced viable pollen *in vitro*. The *in vitro* flowers of *Cymbidium niveo-marginatum* were colored but there is no information on size, organ development completion, or viability of the pollen and ovules.

Doritis pulcherimma x *Kingiella philipinensis*

The effects of media components on flower development were studied in *Doritis pulcherrima* x *Kingiella philippiensis* (*xDoriella* Tiny) plantlets derived from flower stalk explants. The flowers *in vitro* exhibited normal color, size, and appearance when compared to flowers under natural conditions (Duan and Yazawa 1994, 1995b). *In vitro* flowering occurred in plantlets after 6-7 (Duan and Yazawa 1994) or 10-12 (Duan and Yazawa 1995b) months in culture in contrast to 3 years under natural conditions. However, these buds withered unless transferred to a BA-free medium. Other cytokinins failed to produce flower buds. Flowering buds were observed on low-nitrogen media supplemented with 14.4 μM BA after 80 days. The optimal nitrogen concentration was 6-9 mM and a high ratio of NH_4^+/ NO_3^- ions was beneficial. In addition, root removal also stimulated *in vitro* flower bud formation. The authors concluded that BA was essential for flower bud formation but inhibited complete flower development.

Ornithogalum arabicum

The environmental signals involved in flower induction and GA₃ as a substitute for external signals were examined *in vitro* (Halaban et al. 1965). The transition from vegetative to reproductive phase in *Ornithogalum* buds occurs inside the bulb during the season before flowering. This change and the development of the inflorescence that follows require specific thermoperiodic conditions for induction, initiation, and elongation of the inflorescence. The authors found that 16 weeks at 30ºC induced this phase change *in vitro* in excised buds. Inflorescences were developed *in vitro* if the buds were kept for 6 weeks at 20ºC followed by 4 weeks at 13ºC. The rate of inflorescence development *in vitro* increased with shoot tip size (a bigger shoot tip was composed of the apex with attached scale tissue). The scale tissue contributed to the increased rate more than the leaf primordia. The inflorescences were developed on a media with Knop's macro-elements (Knop 1865) and Heller's microelements (Heller 1953), supplemented with 2% sucrose and solidified by 0.8% agar. However, *in vitro* flowering was shown only in buds from flowering-sized bulbs. In this study GA₃ was the only growth regulator tested. GA₃ (0.29 mM) could not stimulate *in vitro* flowering but caused bolting of the meristem and inhibited leaf elongation. No inflorescence differentiation was observed in these apices. The optimal treatment for induction was not given to the bulbs prior to GA₃ application, however.

Panax ginseng (ginseng)

Shortening the long juvenile period by ca. 3 years is advantageous for breeding programs of ginseng (Chang and Hsing 1980). *In vitro* flowering of ginseng was obtained from plantlets developed from either a juvenile source (Lee et al. 1990, 1991, Tang 2000) or from adult plants (Chang and Hsing 1980, Lin et al. 2003a, 2005). *In vitro* flowering where the juvenility period was shortened was obtained directly from zygotic embryos (Lee et al. 1991), or indirectly from somatic embryos, which developed from zygotic embryo calli (Lee et al. 1990, Tang 2000). *In vitro* flowering from adult plant sources was obtained from somatic embryos derived from callus of root pith tissue of mature plants (Chang and Hsing 1980, Lin et al 2005) or amplified from inflorescences that developed from mature plant explants (Lin et al. 2003a). Flowers appeared *in vitro* after 1-1.5 months of culture on half-strength MS, full-strength B5, or MS medium. BA (4.4-5 μM) in the medium was essential for flower development, but the effect was blocked by 5 μM ABA (Lee et al. 1991). BA was successfully replaced by 0.5-4.5 μM TDZ in the presence of 0.3-2.9 μM GA₃ (Lin et al. 2005). GA₃ (5 μM) was necessary in the presence of ABA (Lee et al. 1991). However, Tang (2000) showed a low percentage (less than 6%) of *in vitro* flowering in the presence of 5.8 or 11.6 μM GA₃ only, in plantlets derived from adventitious buds or somatic embryos. This indicates that the role of gibberellin in flower induction or development is not clear. Flower formation *in vitro* was inhibited

by a high concentration of NAA (27 µM). In contrast, a lower dose (5.4 µM) seemed to be ineffective (Lin et al. 2005). The flowers were formed on umbels of elongated axillary branches. They were several times smaller than flowers *in vivo*, but bore well-developed anthers (Lee et al. 1990) with fertile pollen (Chang and Hsing 1980). Immature fruits developed from the flowers (Lee et al. 1990, 1991), which eventually degenerated before maturation. Sterile pollen was observed in inflorescences that were amplified directly from other inflorescences (Lin et al. 2003a).

Zantedescia spp. (calla lily)

Colored *Zantedeschia* is considered a DNP with regard to flowering (Naor and Kigel 2002). However, gibberellin enhances flowering in treated tubers of *Zantedeschia*, as well as in several other Araceae species (Corr and Widmer 1987, Tjia 1989, Henny and Hamilton 1992, Funnell 1993, Henny et al. 1999, Kuehny 2000). Plantlets of four *Zantedeschia* cultivars regenerated in TC, flowered *in vitro* in response to gibberellin treatment as shown in Fig. 2A for the 'Crystal Blush' cultivar (Naor et al. 2004). Following gibberellin treatment microtubers of the 'Calla Gold' cultivar regenerated in TC flowered *in vitro* (Fig. 2B). The pattern of the inflorescence development *in vitro* resembled that *in vivo*. The apex switched from a vegetative to a reproductive phase and developed into a floral stem comprised of a spadix surrounded by a spathe located at the top of a peduncle. In developed inflorescences, female florets located at the base of the primordial spadix could be clearly distinguished from male florets located above them, (Fig 2A**).** However, the inflorescences *in vitro* were smaller, sometimes deformed, and in many cases ceased to develop at an early stage. The inflorescences *in vitro* were enclosed within 3-4 leaf primordia and eventually dried. Inflorescences developed *in vitro* in 80-100% of the plantlets within 30-50 days, only if treated with gibberellin. In this study the plantlets were dipped in 6-600 µM gibberellin solutions, prior to subculture on MS medium, in contrast to other studies in which PGR were supplemented in the medium. Inflorescences developed in response to GA_3, GA_1, and GA_4. A pronounced response to GA treatments occurred in plantlets that regenerated from young established shoot cultures and lasted for 30-40 months of consecutive subculturing. In older cultures, however, the response dissipated. Levels of 0.1-13.3 µM BA alone were not sufficient to induce inflorescence development in *Zantedeschia* plantlets *in vitro*. Nevertheless, BA seemed to interact with GA by enhancing inflorescence development. The transition to flowering in *Zantedeschia* plantlets was solely due to treatments with GA, which was found to be an essential growth regulator for the transition from a vegetative to a reproductive bud. Thus the potential to develop inflorescences *in vitro* exists in vegetative buds of *Zantedeschia* plantlets and is expressed when the plantlets are treated with GA. It was also suggested that meristem competence to switch from a vegetative to a reproductive phase exists in *Zantedeschia* plants, regardless of meristem size or age (Naor et al. 2005).

CONCLUSION

In vitro flowering serves as an important tool to study flower induction and initiation, and floral development. Controlling the environment and media components enables the manipulation of the different variables, that affect these processes. Understanding the physiology of flowering is the first step towards
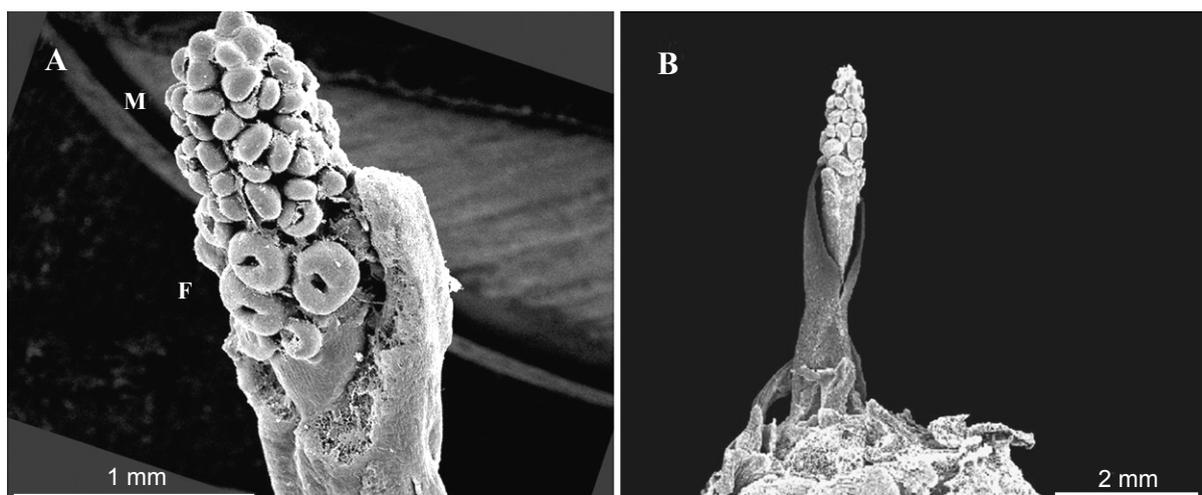

Fig. 2. A) Inflorescence development in the apical bud of colored *Zantadechia* (cultivar 'Crystal Blush') plantlets *in vitro,* treated with 580 µM GA_3, 5-6 weeks after treatment. F- female floret, M- male floret (Naor et al. 2004). B) Inflorescence development (the spadix is enclosed within the spathe on top of the inflorescence peduncle) in the apical bud of colored *Zantadechia* (cultivar 'Calla Gold') microtuber produced *in vitro* and treated with 1.7 mM GA_3, 8 weeks after treatment.

molecular studies of the reproductive phase in plants. However, *in vitro* and *in planta* flowering, which may be similar or different should be closely compared. The TC technique is of practical importance as a breeding tool for species with long juvenile periods, and can also serve for mass production of specific organs with unique compounds for pharmaceutical, nutritional, and other uses.

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